

## **REMARKS**

Claims 1, 12, 13 and 17-50 are currently pending in this application. Claims 12, 13, 18, 19, 29-31, and 41-49 stand withdrawn. Claims 1, 21, 23, 28, 34 and 50 are currently amended. Claims 17, 20, 22, 26, 27, 32 and 37 are cancelled without prejudice or disclaimer as to the subject matter thereof. Claims 2-11 and 14-16 were previously cancelled without prejudice or disclaimer as to the subject matter thereof. Applicants respectfully reserve the right to prosecute the subject matter of the cancelled claims in one or more Continuation or Divisional applications.

### **Claim Amendments**

Applicants respectfully submit that no new matter is introduced into the application by way of the instant claim amendments. Support for the term “immunogenic composition” is found throughout the specification, including the following disclosures:

Page 14, lines 24-26 of the specification, which states that “[a]s the peptides of the invention are relatively small molecules it may be required in such compositions to combine the peptides with various materials such as adjuvants, to produce vaccines, immunogenic compositions, etc.” (emphasis added).

Page 16, lines 1-4 of the specification, which states that “[i]n preferred embodiments, the pharmaceutical composition of the invention is an immunogenic composition or vaccine capable of eliciting an immune response to a cancer disease” and that “[a]s used herein, the expression ‘immunogenic composition or vaccine’ refers to a composition eliciting at least one type of immune response directed against cancer cells.” (emphasis added)

## **Rejections**

### ***35 U.S.C. § 112, 1<sup>st</sup> Paragraph, Written Description***

Claims 1, 17, 20-28, 32-40 and 50 were rejected under 35 U.S.C. § 112, 1<sup>st</sup> paragraph as allegedly failing to comply with the written description requirement.

Applicants respectfully disagree and traverse this rejection. Without acquiescing in the merits of the rejection, it is noted that for purposes of expediting prosecution Applicants have amended claims 1 and 20 herein to direct the claims to subject matter corresponding to SEQ ID NO:5 (“the Sur1M2 peptide”). Accordingly, the arguments presented herein focus on the currently claimed subject matter corresponding to SEQ ID NO:5.

The Office Action states (in part) that “[t]he specification ... does not disclose the relevance of the treatment with the clinical outcome observed, i.e., how the composition or vaccine comprising the peptides treat or prevent cancer.” See Office Action, page 5, lines 23-29.

Applicants respectfully disagree with this statement. In fact, the specification not only does describe clinical benefits in all patient receiving the full treatment, it also reveals that this treatment is accompanied by a highly specific T-cell response to the Sur1M2 peptide presented in the vaccine.

### **Therapeutic benefits**

Example 5, entitled “Therapeutic Trial Procedures Using Survivin-Derived Peptides as Immunogens,” describes positive clinical responses in all four late stage melanoma patients receiving full therapy during the trial:<sup>1</sup>

“In one patient (WW), preexisting liver metastases could be stabilized under vaccination therapy ... The other three patients [GB, KN, RW] demonstrated slow progression of metastatic disease

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<sup>1</sup> “Due to the manifestation of symptomatic brain metastases, one patient was taken off therapy after only two vaccinations.” See specification, page 46, lines 10-12. “The other four patients received up to 15 vaccinations.” Id.

without substantial impairment in their general state of health. Remarkably, for patient KN, an overall survival of 13 months (from vaccination start to death) could be achieved despite a heavy metastatic load and fast disease progression at the start of vaccination."

See specification, page 46, lines 23-31 (emphasis added).

Example 5 further indicates that no significant side effects or adverse reactions occurred in these four patients:

No major toxicities occurred. Thus, hemoglobin, leucocytes and thrombocytes, as well as lactate dehydrogenase, creatinine and cholinesterase were not influenced by the vaccination therapy (FIG. 15). No signs of systemic or local toxicity were observed at the injection sites. Furthermore, there was no detection of impaired wound healing, hemorrhagic disorders, cardiac dysfunction, vasculitis or inflammatory bowel disease.

See specification, page 46, lines 18-22 and Figure 15.

#### Specificity of T-cell response

At least three observations indicate that the observed T cell response during treatment is highly specific to the Sur1M2 peptide:

First, the T cell response is *temporally* specific. "FIG. 16 demonstrates kinetic analysis of immunity to survivin peptides assessed by IFN $\gamma$  ELISPOT." See specification, page 20, lines 23-24. In this study, "PBMCs were obtained before the first DC vaccination and three months thereafter" and "[t]he number of IFN $\gamma$  spot-forming cells above background ..." was determined. See specification, page 20, lines 24-25 (*emphasis added*). Applicants submit that FIG. 16 shows that the number of IFN- $\gamma$  spot-forming cells above background was much lower, if not detectable before vaccination. In other words, the T cell response to survivin peptides was only seen after vaccination.

Second, the T cell response is *molecularly* specific. "[I]n all four patients tested, an induction of survivin<sub>96-104</sub> reactive T cells was evident (FIG. 16)." See specification, page 46,

lines 42-43. In contrast, a T cell responses to survivin peptides that were not present in the vaccine was not uniformly seen in the patients. *See* specification, page 46, line 43 extending to page 47, line 2. The only peptide eliciting a specific T cell response in all four patients was the Sur1M2 peptide presented by the autologous dendritic cells.

Third, the T cell response is *spatially* specific. Sur1M2-specific T-cell responses were first visualized at the vaccination site and subsequently in soft tissue and visceral metastases:

The prognostic and clinical value of measurements of tumor-specific T-cell responses in peripheral blood has been questioned repeatedly; thus, we also tested for the presence of survivin<sub>96-104</sub>/HLA-A\*0201 reactive CD8+ T lymphocytes among tumor infiltrating lymphocytes in situ by peptide/MHC multimer staining. To validate the method, we first analyzed tissue samples from delayed type hypersensitivity reactions occurring at the vaccination site within 24 hours. This analysis confirmed earlier observations that intradermal injections of peptide-pulsed DC *induce a strong peptide specific* inflammatory T-cell infiltrate. Subsequently, the peptide/MHC multimer staining procedure was applied on soft tissue and visceral metastases, which revealed the presence of survivin<sub>96-104</sub>/HLA-A\*0201 reactive cells among the CD8+ infiltrate.

*See* specification, page 47, lines 4-13.

In sum, the specification provides evidence that vaccination therapy based on the claimed Sur1M2 peptides, presented by dendritic cells, is associated with distinct clinical benefits, is well tolerated, and is accompanied by a highly specific T-cell response to the vaccine antigen.

In light of the instant amendments and remarks provided herein, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 20-28, 32-40 and 50 under 35 U.S.C. § 112, 1<sup>st</sup> paragraph as allegedly failing to comply with the written description requirement. Applicants note that rejected claims 17, 26, 27 and 32 are cancelled herein without prejudice or disclaimer.

**35 U.S.C. § 112, 1<sup>st</sup> Paragraph, Enablement**

• **Claims 1, 17, 20-28, 32-40 and 50**

Claims 1, 17, 20-28, 32-40 have been rejected under 35 U.S.C. § 112, 1<sup>st</sup> paragraph as allegedly failing to provide enablement commensurate with the scope of the claims.

Applicants respectfully disagree and traverse this rejection. Without acquiescing in the merits of the rejection, it is noted that for purposes of expediting prosecution Applicants have amended claims 1 and 20 herein to direct those claims to the subject matter of SEQ ID NO:5, which represents the Sur1M2 peptide.

Under 35 U.S.C. §112 ¶ 1, the “enablement requirement is satisfied when one skilled in the art, after reading the specification, could practice the claimed invention without undue experimentation.”<sup>2</sup> “The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation ‘must not be unduly extensive.’”<sup>3</sup>

The Examiner’s position appears to be that the specification enables peptides and kits (and HLA-A2 complexes or multimers) but not vaccines and pharmaceutical compositions:

“... (2) Examples 2 and 5 do not disclose a clinical response for treatment or prevention using SEQ ID NO:5, and Example 5 discloses that a T cell response could be elicited when dendritic cells were pulsed with SEQ ID NO:5 and administered *in vivo* and T cell infiltration of metastases could be observed, and the instant rejection does not acknowledge enablement for SEQ ID NO:5 except for the peptide consisting of SEQ ID NO:5, kit thereof, HLA-A2/complex or multimer thereof, not for the vaccine, pharmaceutical composition or other compositions as enunciated supra, ... (4) Dr. Andersen confirms that the only firm proof that a peptide is a vaccine or pharmaceutical is administration in phase III clinical trials that have not been completed ...”

See Office Action, page 14, line 34 to page 15, line 13.

<sup>2</sup> *AK Steel Corp. v. Sollac & Ugine*, 344 F.3d 1234, 1244 (Fed. Cir. 2003); see also *In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988).

<sup>3</sup> *PPG Indus., Inc. v. Guardian Indus., Corp.*, 75 F.3d 1558, 1564, U.S.P.Q.2d 1618, 1623 (Fed. Cir. 1996) (quoting *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984)).

(continued...)

Applicants respectfully submit the scope of the claims should not be so limited because the Examiner's position is misplaced for at least two reasons:

1.) Clinical trial data

First, the Examiner appears to require completed human clinical trials to satisfy the enablement (and by extension the utility) requirement. However, the Federal Circuit has ruled that such a requirement is improper:

FDA approval is not a prerequisite for finding a compound useful within the meaning of the patent laws. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.<sup>4</sup>

In accord with this ruling, the M.P.E.P. states that "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials ... Thus, as a general rule, if an applicant has initiated human clinical trials for a therapeutic product or process, Office personnel should presume that the applicant has established that the subject matter of that trial is reasonably predictive of having the asserted therapeutic utility."<sup>5</sup>

In other words, the disclosure of clinical data in the instant case is presumptive evidence that *supports* -- not undermines -- enablement of the claimed vaccine and pharmaceutical compositions. Regarding the Reker *et al* reference, Dr. Andersen explained that the "statement was presented in the discussion of the research data, not to express concerns with respect to the

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<sup>4</sup> *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1437, 1442-1443 (Fed. Cir. 1995).

<sup>5</sup> MPEP § 2107.03 (IV)( "Human Clinical Data") (8th Ed., 7th Rev.) (emphasis in original).

relevance of using survivin peptides in cancer immunotherapy, but merely to indicate that phase III clinical trials - the only firm proof that a vaccine works - had not yet been completed.”<sup>6</sup>

2.) In vitro-in vivo correlation

Second, the M.P.E.P. states that “... data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process” so long as the data is reasonably correlated to the asserted utility.<sup>7</sup> Under this standard, “[t]he applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor does he or she have to provide actual evidence of success in treating humans where such a utility is asserted.”<sup>8</sup>

The instant application meets this standard because the Examples disclose data from multiple *in vitro* assays that adequately establish therapeutic utility of the claimed Sur1M2 peptide compositions—including immunogenic compositions and vaccines:

Example 1 of the instant application provides data from *in vitro* assays measuring binding of the Sur1M2 peptide to Class I MHC molecules and quantifying its ability to induce a Cytotoxic T-Lymphocyte (CTL) response. The results reveal high-affinity binding of Sur1M2 peptide to HLA-A2 ( $C_{50} = 1 \mu\text{M}$ ) — a level similar to the positive control. *See* specification, page 25, lines 13-16. In addition, the Sur1M2 peptide induced a strong CTL response in peripheral blood isolated from a cancer patient, as quantified by the ELISPOT assay. *See* specification, page 25, lines 20-41. Applicants submit that the results suggest that the Sur1M2 peptide can form a stable complex on an antigen-presenting cell, such as a dendritic cell, and is able to induce a strong CTL response.

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<sup>6</sup> *See* Declaration under 37 C.F.R. § 1.132 of Dr. Mads Hald Andersen, of record.

<sup>7</sup> M.P.E.P. § 2107.03(III) ( “Data From *In Vitro* or Animal Testing ...”) (8th Ed., 7th Rev.).

<sup>8</sup> Nelson v. Bowler, 626 F.2d 853, 857, 206 U.S.P.Q. 881, 884 (C.C.P.A. 1980); M.P.E.P. 2107.03(I).

Confirming and extending these observations, Example 2 provides data from multiple assays, including immunohistochemistry and cytotoxicity studies. *In situ* staining revealed HLA-A2/Sur1M2-reactive CTLs in the primary tumor and the sentinel lymph node of a stage III melanoma patient as well as in a primary breast cancer lesion. Moreover, HLA-A2/Sur1M2-reactive CTLs isolated from a melanoma infiltrated lymph node effectively lysed certain HLA-A2 positive melanoma and HLA-A2 positive breast cancer cell lines. In contrast, these CTLs did not lyse certain HLA-A2 negative melanoma and HLA-A2 negative breast cancer cell lines. See specification, page 28, lines 1-32 and Figure 5. Applicants submit that these results provide further evidence that compositions based on the Sur1M2 epitope will be immunogenic and able to induce highly specific CTLs capable of killing tumor target cells.

Further support for the enablement of the claims of the instant application is found in the Sørensen *et al.* manuscript that has been accepted for publication (attached herewith as Appendix B)<sup>9</sup>. HLA-A2/Sur1M2-reactive CTLs were isolated (in this case from a HLA-A2 breast cancer patient), but in a further step the authors isolated and expanded certain CTL clones. Corroborating the results disclosed in the instant application, an individual CTL clone was tested and shown to be able to lyse a large panel of tumor cells of different origins. These cells include certain breast cancer and melanoma cells, as in the instant application, as well as colon cancer cells. These findings confirm the molecular specificity and functional efficacy of HLA-A2/Sur1M2-reactive CTLs.

Example 5 discloses therapeutic trial procedures using immunogenic compositions, and in particular, vaccines in which the Sur1M2 peptide is presented by autologous dendritic cells. Coincident with clinical observations, these studies present data from several *in vitro* assays to analyze the temporal, molecular, and spatial specificity of the CTL responses. (This data was described above in the sub-section entitled “Specificity of T-cell response”). Briefly, these assays showed that “... vaccination does not only induce T cells with the desired [Sur1M2]

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<sup>9</sup> The Sørensen *et al.* manuscript has been accepted for publication in Cancer Biology and Therapy (See <http://www.landesbioscience.com/journals/cbt/article/6935/>)

specificity, but it also endows them with the necessary homing capacity,” (i.e., to “soft tissue and visceral metastases.”) See specification, page 47, lines 4-15.

In sum, Applicants submit that the extensive *in vitro* data disclosed in the instant application provides evidence that the Sur1M2 peptide can bind with high-affinity to an MHC 1 molecule and induce a specific and strong CTL response that can infiltrate cancerous tissue. These attributes correlate with the claimed therapeutic utility of a Sur1M2 cancer vaccine in inducing highly specific CTLs capable of homing to and attacking tumor cells. Accordingly, the specification supports the full scope of uses encompassed by the claimed subject matter.

The instant specification not only supports the full scope of claimed uses for the Sur1M2 peptide, it also provides detailed guidance regarding formulating, administering, and testing the pharmaceutical compositions, including immunogenic compositions and vaccines. (See, e.g., Example 5). This specific guidance, combined with standard knowledge in the art, enables one of ordinary skill to practice the full scope of the claimed methods and compositions without undue experimentation.

Accordingly Applicants submit that the scope of claims in the instant application is reasonably commensurate with the scope of the enabling disclosure.<sup>10</sup> Applicants further submit that the evidentiary references cited by the Examiner do not counter this conclusion. For example, the Examiner appears to cite Matthias *et al.*<sup>11</sup> as evidence that the induced Sur1M2-specific CTL response in Example 5 results from dendritic cells *alone* in the vaccine. More particularly, the Examiner cites the observation in Matthias *et al.* that survivin-peptide-specific CTLs could be detected in 5% of healthy volunteers.

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<sup>10</sup> See *In re Wright*, 999 F.2d 155, 27 U.S.P.Q.2d 1510 (Fed. Cir. 1993) (“The scope of the claims must bear a reasonable relationship to the scope of enablement.”).

<sup>11</sup> Grube, M. *et al.*, Blood, 196, part 2, pp. 369B (2005).

Matthias *et al.* is an abstract. Recently published Matthias-II (attached herewith as Appendix A) is believed to be associated with the cited Matthias abstract.<sup>12</sup> Using quantitative PCR, the authors in Matthias-II detected survivin-specific T-cells (including CD8+) responses in 9 of 23 patients (39%) but in only 1 of 21 healthy volunteers (4.7%). It is noteworthy in Matthias-II that the 4.7% survivin-specific T-cell response in healthy volunteers corresponded to only one healthy volunteer.

The results in the instant application reveal robust stimulation of Sur1M2-specific CTLs in all four patients tested. Moreover, this observation is consistent with the conclusion reached in Matthias-II: that "... survivin could serve as useful target antigen for T cell-based immunotherapeutic strategies in the treatment of multiple myeloma." See Matthias-II, page 1060, 1<sup>st</sup> col., lines 4-6. Therefore, Applicants submit that at least Matthias-II supports enablement of the claims in the instant application.

More generally, the proof of concept of vaccination therapy using peptide-pulsed dendritic cells has already been demonstrated in treating multiple myeloma. In Titzer *et al.*,<sup>13</sup> (attached herewith as Appendix C) an article cited in Matthias-II, patients with advanced multiple myeloma were treated with a vaccine based on dendritic cells pulsed with "Id" peptides (corresponding to a myeloma specific antigen). An Id-specific T-cell response was observed in four out of 10 patients after vaccination. The treatment was well tolerated with no side effects, and there was no comparable T-cell response following vaccination with a control Id peptide.

In light of the instant amendments and remarks provided herein, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 20-28, 32-40 and 50 under 35 U.S.C. § 112, 1<sup>st</sup> paragraph. Applicants note that rejected claim 17, 26, 27 and 32 are cancelled herein without prejudice or disclaimer.

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<sup>12</sup> Grube, M. *et al.* CD8+ T cells Reactive to Survivin Antigen in Patients with Multiple Myeloma, Clin. Cancer Res., 13:1053-1060 (2007).

<sup>13</sup> Titzer S, *et al.*, Vaccination of multiple myeloma patients with idiotype-pulsed dendritic cells: immunological and clinical aspects. Br. J. Haematol., 108:805-16 (2000).

- **Claim 24**

Claim 24 was rejected under 35 U.S.C. § 112, 1<sup>st</sup> paragraph as allegedly failing to comply with the enablement requirement. The Office Action asserts that there is insufficient disclosure in the specification on the breast cancer cell line MCF-7 and melanoma cell line FM3.

Applicants respectfully disagree and traverse.

Applicants submit that the breast cancer cell line MCF-7 is commercially available from the LGC Promochem / ATCC as ATCC Number HTB-22 <sup>TM</sup>, and thus it is not an undue burden on the skilled artisan to obtain this cell line. (See <http://www.lgcpromochem-atcc.com/common/catalog/numSearch/numResults.cfm>).

Regarding the melanoma cell line FM3, this is also commercially available from the ESTDAB database. (See [http://www.ebi.ac.uk/cgi-bin/ipd/estdab/print\\_cell.cgi?ESTDAB-007](http://www.ebi.ac.uk/cgi-bin/ipd/estdab/print_cell.cgi?ESTDAB-007)). Furthermore, the terms for ordering cells from the database are explained at: <http://www.ebi.ac.uk/ipd/estdab/ordercells.html>. Applicants reiterate that Dr. Andersen states in his declaration of record that “[t]he cell line was originally described by Kirkin *et al.* (Cancer Immunol Immunother, 41:71-81 1995) and is well recognized within the art.”

Applicants respectfully request reconsideration and withdrawal of the rejection of claim 24 under 35 U.S.C. § 112, 1<sup>st</sup> paragraph.

**35 U.S.C. § 112, 2<sup>nd</sup> paragraph**

- **Claim 20**

Claim 20 was rejected as indefinite in the recitation of “[a] peptide ... comprising, for each specific HLA allele, any of the amino acid residues as indicated in the following table ...” Applicants submit that this rejection has been rendered moot by way of claim amendment, and respectfully request reconsideration and withdrawal of the rejection of claim 20 as indefinite.

- **Claim 24**

Claim 24 was rejected as indefinite in the recitation of "... the breast cancer cell line MCF-7 and the melanoma cell line FM3." Applicants submit that the recitation of breast cancer cell line MCF-7 and melanoma cell line FM3 is not indefinite, as these are art recognized cell lines. For example, the breast cancer cell line MCF-7 is commercially available from the LGC Promochem / ATCC as ATCC Number HTB-22 <sup>TM</sup>, and thus it is not an undue burden on the skilled artisan to obtain this cell line. (See <http://www.lgcpromochem-atcc.com/common/catalog/numSearch/numResults.cfm>).

Regarding the melanoma cell line FM3, this is also commercially available from the ESTDAB database. (See [http://www.ebi.ac.uk/cgi-bin/ipd/estdab/print\\_cell.cgi?ESTDAB-007](http://www.ebi.ac.uk/cgi-bin/ipd/estdab/print_cell.cgi?ESTDAB-007)). Furthermore, the terms for ordering cells from the database are explained at: (See <http://www.ebi.ac.uk/ipd/estdab/ordercells.html>). Applicants reiterate that Dr. Andersen states in his declaration of record that "[t]he cell line was originally described by Kirkin et al (Cancer Immunol Immunother, 41:71-81 1995) and is well recognized within the art."

### **Prior Art Rejections**

- **Andersen References**

As an initial matter, Applicants respectfully address the characterization of both of the cited Andersen references as prior art. Applicants submit that the earliest priority filing (U.S. provisional patent application no. 60/352,284) was filed on January 30, 2002, and includes disclosure of at least SEQ ID NO:5, to which amended claim 1 is now directed. Furthermore, exemplary support for each of the following amended claims may be found in at least the following text of the priority filing:

<b>Current Claim</b>	<b>Exemplary Support in Priority Filing</b>
1	p. 3, Table 1; p. 11, lines 5-18; and p.12, lines 15-35
21	p. 4, lines 5-15; p. 5, lines 12-26; and Figure 2
23	p. 2, lines 18-19; p. 7, lines 26-29; p. 9, lines 9-14 and 23-31; and p. 14, lines 29-39
24	p. 10, line 39 extending to p. 11, line 2; p. 11, lines 40 extending to p. 12, line 12
25	p. 24, lines 1-3
28	p. 23, line 42 extending to p. 24, line 7
33	p. 3, Table 1; p. 11, lines 5-18; and p.12, lines 15-35
34	p. 2, lines 18-19; p. 7, lines 26-29; p. 9, lines 9-14 and 23-31; and p. 14, lines 29-39
35	p. 3, Table 1; p. 11, lines 5-18; and

	p.12, lines 15-35
36	p. 23, lines 35-41
38	p. 23, lines 31-33
39	p. 23, lines 31-33
40	p. 23, lines 31-33
50	p. 23, line 42 extending to p. 24, line 7

Applicants further submit that both Andersen references were published less than one year prior to the filing of Applicants' provisional patent application, for the reasons of record previously presented by Applicants. Because Applicants' provisional application was filed less than one year after the publication of the two Andersen references, Applicants submit that the Andersen references are not prior art under 35 U.S.C. § 102(b).

Furthermore, Applicants have previously submitted a declaration under 37 C.F.R. § 1.132 by inventor Mads Hald Andersen (of record), in which Dr. Andersen sets forth the contribution of each of the non-inventor and inventor authors of the two cited Andersen publications. Applicants submit that the declaration of Dr. Andersen establishes that the cited Andersen *et al* articles are describing Applicants' own work.

- **35 U.S.C. § 102 (b)**

a) Claims 1, 17, 20-24, 36 and 38-40 were rejected under 35 U.S.C. § 102 (b), as allegedly anticipated by the disclosure of Andersen *et al* (Cancer Res., 2000, 61:869-872) as evidenced by Andersen *et al* (Cancer Res., 2001, 61:5964-5968).

Applicants respectfully disagree and traverse this rejection.

As discussed above, Applicants believe that neither of the cited Andersen *et al* references is prior art under 35 U.S.C. § 102 (b) to the claimed invention for the reasons provided. Furthermore, Applicants submit that the declaration of Dr. Andersen under 37 C.F.R. § 1.132 establishes that the Andersen *et al* articles are describing Applicants' own work. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 17, 20-24, 36 and 38-40 under 35 U.S.C. § 102 (b).

b) Claims 1, 17, 20-24, 36 and 38-40 were rejected under 35 U.S.C. § 102 (b), as allegedly anticipated by the disclosure of Andersen *et al* (Cancer Res., 2001, 61:5964-5968).

Applicants respectfully disagree and traverse this rejection.

As discussed above, Applicants believe that neither of the cited Andersen *et al* references is prior art under 35 U.S.C. § 102 (b) to the claimed invention for the reasons provided. Furthermore, Applicants submit that the declaration of Dr. Andersen under 37 C.F.R. § 1.132 establishes that the Andersen *et al* article is describing Applicants' own work. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 17, 20-24, 36 and 38-40 under 35 U.S.C. § 102 (b).

c) Claims 1, 17, 20-24, 36 and 38-40 were rejected under 35 U.S.C. § 102 (b), as allegedly anticipated by the disclosure of U.S. Patent No. 6,346,389, for the alleged disclosure of a subsequence corresponding to SEQ ID NO:14.

Applicants respectfully disagree and traverse this rejection.

As stated in MPEP § 2131, "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference."<sup>14</sup>

Applicants submit that the disclosure of U.S. Patent No. 6,346,389 fails to teach all of the claimed elements. For example, U.S. Patent No. 6,346,389 fails to teach SEQ ID NO:5. As noted previously, Applicants submit that the declaration of Dr. Andersen under 37 C.F.R. § 1.132 establishes that the Andersen *et al* article is describing Applicants' own work. As U.S. Patent No. 6,346,389 does not teach all of the elements of the rejected claims as amended herein, U.S. Patent No. 6,346,389 does not anticipate the subject matter of the rejected claims. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 17, 20-24, 36 and 38-40 under 35 U.S.C. § 102 (b).

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<sup>14</sup> Verdegaal Bros. v. Union Oil Co. of California, 814 F.2d 628, 631 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

d) Claims 1, 17, 20-24, 36 and 38-40 were rejected under 35 U.S.C. § 102 (e), as allegedly anticipated by the disclosure of U.S. Patent No. 6,346,389, for the alleged disclosure of a subsequence corresponding to SEQ ID NO:14.

Applicants respectfully disagree and traverse this rejection.

As stated in MPEP § 2131, “[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.”<sup>15</sup>

Applicants submit that the disclosure of U.S. Patent No. 6,346,389 fails to teach all of the claimed elements. For example, U.S. Patent No. 6,346,389 fails to teach SEQ ID NO:5. As noted previously, Applicants submit that the declaration of Dr. Andersen under 37 C.F.R. § 1.132 establishes that the Andersen *et al* article is describing Applicants’ own work. As U.S. Patent No. 6,346,389 does not teach all of the elements of the rejected claims as amended herein, U.S. Patent No. 6,346,389 does not anticipate the subject matter of the rejected claims. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 17, 20-24, 36 and 38-40 under 35 U.S.C. § 102 (e).

• **35 U.S.C. § 103(a)**

a) Claims 1, 17, 20-25, 28 and 32-37 were rejected under 35 U.S.C. § 103(a) as allegedly obvious over WO 00/03693 in view of Rammensee *et al.*, Ruppert *et al.*, Conway *et al.* and U.S. Patent No. 6,572,864.

Applicants respectfully disagree and traverse this rejection.

According to the Office Action, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention in light of WO 00/03693, Conway *et al.*, Rammensee *et al.* and Ruppert *et al.* “in order to produce the nonamer peptide sequence FLKLDREIRA (SEQ ID NO:1) and the nonamer subsequence STFKNWPFL (SEQ ID NO:14).” See Office Action, page 22, lines 15-22. The currently amended claims are not directed to SEQ ID NO:1 or SEQ ID NO:14. Applicants respectfully submit that the amended claims are not

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<sup>15</sup> Id.

obvious in light of the disclosures of WO 00/03693, Conway *et al.*, Rammensee *et al.*, Ruppert *et al.* and US Patent No. 6,572,864. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 17, 20-25, 28 and 32-37 under 35 U.S.C. § 103(a).

b) Claims 38-40 were rejected under 35 U.S.C. § 103(a) as allegedly obvious over WO 00/03693 in view of Rammensee *et al.*, Ruppert *et al.*, Conway *et al.*, U.S. Patent No. 6,572,864, and further in view of WO 99/50637.

Applicants respectfully disagree and traverse this rejection.

Claims 38-40 ultimately depend from and incorporate the elements of claim 1. Applicants submit that claim 1 is not rendered obvious by these references for the reasons set forth above. Applicants respectfully submit that claims 38-40 are also not obvious for at least the reasons set forth above with respect to claims 1, 17, 20-25, 28 and 32-37. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 38-40 under 35 U.S.C. § 103(a).

### **CONCLUSION**

An indication of allowance of all claims is respectfully solicited. Early notification of a favorable consideration is respectfully requested.

Respectfully submitted,

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## **Appendix A**

## CD8<sup>+</sup> T cells Reactive to Survivin Antigen in Patients with Multiple Myeloma

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**Abstract** Purpose: Survivin is a member of the inhibitors of apoptosis family and is overexpressed in different types of malignancies. Cytotoxic T cells recognizing survivin epitopes can be elicited *in vitro* and by vaccination in patients with leukemia, breast cancer, and melanoma. We did this study to investigate whether survivin-specific CD8<sup>+</sup> T cells occur in patients with multiple myeloma.

**Experimental Design:** An HLA-A2.1-binding survivin peptide was used to detect peptide-specific T cells by a quantitative real-time PCR to measure antigen-specific IFN- $\gamma$  mRNA expression in 23 patients with myeloma and 21 healthy volunteers. T cells producing IFN- $\gamma$  in response to survivin were further analyzed for expression of CD45RA and CCR7 to determine phenotypic characterization. Additional immunohistochemical analyses of survivin antigen expression in bone marrow specimens of patients was done.

**Results:** T cells recognizing HLA-A2.1-binding survivin peptide were detected in 9 of 23 patients and in 1 of 21 healthy volunteers. Survivin-reactive T cells were identified as terminally differentiated effector T cells (CD8<sup>+</sup>, CD45RA<sup>+</sup>, and CCR7<sup>-</sup>). Positive survivin expression of myeloma cells in bone marrow specimens was shown in 7 of 11 patients.

**Conclusion:** We provide, for the first time, evidence of T cell reactivity against survivin antigen in patients with multiple myeloma. Our data suggest the immunogenicity of survivin antigen in multiple myeloma and that immunotherapeutic strategies using survivin as a target antigen might be an option for patients with this disease.

Multiple myeloma represents a malignant proliferation of plasma cells derived from a single clone characterized by secreting monoclonal immunoglobulins with a concomitant decrease in normal immunoglobulins and lytic bone lesions (1). It represents the second most frequent hematologic malignancy in the U.S. and accounts for 10% of all such diseases. Despite the considerable progress in understanding its biology and therapy, the disease has essentially remained incurable. The current standard of treatment consists of high-dose myeloablative chemotherapy followed by autologous peripheral stem cell transplantation (PBSZT), and has significantly

improved survival of patients compared with conventional chemotherapy (1). However, molecular remission is rare and most of the patients relapse due to minimal residual disease. Allogeneic transplantation and donor lymphocyte infusion offer a curative potential due to the graft-versus-myeloma effect exerted by T lymphocytes, but are associated with a high transplant-related mortality (2). Recent efforts to improve the safety of transplant procedures with reduced intensity or a nonmyeloablative conditioning regimen have shown encouraging results, but longer follow-up is needed to determine the role of this treatment modality (3).

Because current treatment strategies are not expected to offer a cure or long-term survival without considerable toxicity, the development of new therapeutic strategies is required. Recently, several studies have focused on the identification of T-cell target epitopes that are expressed on myeloma cells (4) and on the development of immunotherapeutic strategies (e.g., vaccination and generation of antigen-specific cytotoxic T cells, CTL) to augment the patients' immune response to eliminate neoplastic cells (5-7).

Survivin, a member of the inhibitor of apoptosis gene family, has recently been suggested as a promising target antigen for immunotherapeutic approaches in different malignancies (8-11) and has not yet been examined in multiple myeloma. It is present during normal fetal development but is undetectable in most terminally differentiated adult tissues except thymus cells, CD34<sup>+</sup> bone marrow-derived hematopoietic progenitor cells, basal colonic epithelial cells, and activated endothelial

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cells (12). Functionally, survivin is involved in the control of apoptosis (by inhibition of caspase activity) and the regulation of cell division (11). Survivin is highly expressed in most human cancer cells of epithelial and hematopoietic origin, and overexpression is associated with cancer progression, poor prognosis, resistance, and short patient survival (8, 9, 13, 14). Several survivin epitopes could be identified recently, capable of inducing specific cytotoxic T-cell responses in patients with leukemia, breast cancer, and melanoma (15–18). Besides cellular reactivity, the existence of antibodies recognizing survivin antigen has been shown in lung and colorectal cancer (19, 20). A clinical vaccination study using survivin peptide-pulsed dendritic cells has been recently done in melanoma and did not show major toxicities (e.g., autoimmune reaction) after immunization (21).

Recently, it has been shown that survivin seems to play a critical role in the survival and proliferation of human myeloma cells (22, 23) and that *in vitro*-induced survivin-specific cytotoxic T cells from healthy individuals have the ability to lyse multiple myeloma cell lines *in vitro* (16). The fact that enhanced expression of survivin is almost completely restricted to malignant tissues, and that manipulating the survivin-mediated antiapoptotic pathway seems to impair tumor cell proliferation (24–27), makes survivin an interesting target molecule for immunotherapeutic strategies.

In this study, we investigated whether CD8<sup>+</sup> T-cell responses against survivin epitopes occur in patients with multiple myeloma. We detected CD8<sup>+</sup> T cells recognizing a recently described HLA-A2.1-binding peptide in a significant proportion of patients with multiple myeloma and identified them as terminally differentiated effector T cells by analyzing phenotypic expression of CD45RA and CCR7. Additional immunohistochemical staining of bone marrow specimens of patients confirmed survivin expression in neoplastic cells in most of the patients analyzed. In conclusion, we show for the first time that terminally differentiated effector T cells recognizing survivin antigen circulate in patients with multiple myeloma providing evidence for the potential use of survivin as target molecule for immunotherapeutic approaches in multiple myeloma.

## Materials and Methods

**Patients and healthy controls.** After informed consent, cells from HLA-A2-positive patients (which were enrolled in a clinical investigation approved by the institutional ethics committee) with multiple myeloma ( $n = 23$ ) and from healthy individuals ( $n = 21$ ) were obtained from the peripheral blood. One of the patients (patient 4) had an additional history of ovarian cancer, which was in complete remission at the time of analysis. Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque gradient density (PAA Laboratories, Coelbe, Germany) and subsequently frozen in RPMI 1640 complete medium (25 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin; Life Technologies, Karlsruhe, Germany) containing 20% heat-inactivated FCS and 10% DMSO according to the standard protocols. Before use, cells were thawed, washed, and resuspended in complete medium supplemented with 10% human AB serum and rested overnight.

**HLA typing.** The expression of HLA-A2 in patients and healthy individuals was determined by using phycoerythrin-conjugated mouse anti-human monoclonal antibodies (BB7.2; Serotec, Düsseldorf, Germany). Data acquisition was done on FACSCalibur and analyzed using CellQuest software (Becton Dickinson, Heidelberg, Germany).

**Cell lines.** C1RA2 cells are a MHC class I-defective LCL cell line that expresses a transfected genomic clone of HLA-A2.1 (28). The cells were maintained in complete medium supplemented with 10% FCS and used as antigen-presenting cells.

**Peptide synthesis.** The following peptides were used in this study: survivin<sub>105-104</sub> (ELTLGELFLK, ref. 15), gp100<sub>209-217</sub> (2M; IMDQVFFSV), MUC1A<sub>12-20</sub> (LLLTLLTVT), and MHC1B<sub>950-958</sub> (STAPPVHNQ; ref. 29). The peptides were synthesized by ProImmune (Oxford, United Kingdom) to a minimum of 95% purity as measured by high performance liquid chromatography, dissolved in DMSO at a concentration of 5 mg/mL, further diluted in PBS and stored at  $-20^{\circ}\text{C}$ .

**T cell *in vitro* stimulation.** To determine peptide-specific CD8<sup>+</sup> T-cell reactivity, we measured the IFN- $\gamma$  mRNA gene expression by CD8<sup>+</sup> T cells stimulated with candidate peptides. T cells were analyzed without *in vitro* expansion. Multiple experiments to optimize assay conditions were done previously (30–32). Cryopreserved or fresh PBMC ( $1 \times 10^6$ ) were plated in a 96-well flat-bottomed plate in 200  $\mu$ L of complete medium supplemented with 10% human serum and incubated overnight at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  to minimize background expression of IFN- $\gamma$  mRNA expression due to lymphocyte manipulation. PBMC were then stimulated *in vitro* with peptides using an adapted protocol from previous studies (33). Briefly, C1RA2 cells (as APC) were washed thrice in serum-free complete medium and incubated with the test peptide at 10  $\mu$ g/mL in complete medium at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 2 h. The peptide-loaded cells were then irradiated with 7,500 cGy, washed once, suspended in complete medium containing human serum, and added to the isolated PBMC at a 1:1 ratio. As controls, PBMC were either incubated with unloaded C1RA2 cells (negative control), or with C1RA2 cells and 5  $\mu$ g/mL of staphylococcus enterotoxin B (Sigma-Aldrich, Munich, Germany; positive control). After 3 h of cocultivation at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ , cells were harvested for RNA isolation and cDNA synthesis. As additional negative controls, PBMC were incubated with C1RA2 cells pulsed with gp100 (209-2M) as irrelevant peptide.

**RNA extraction and cDNA synthesis.** Total RNA was isolated from test samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at  $-80^{\circ}\text{C}$ . For cDNA synthesis, 1  $\mu$ g of total RNA was reverse-transcribed into cDNA with the Reverse Transcription System cDNA Kit (Promega, Mannheim, Germany) and stored at  $-20^{\circ}\text{C}$ .

**Quantitative real-time PCR.** Measurement of IFN- $\gamma$  mRNA gene expression was done using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA) as previously described (34, 35). The feasibility of this approach for the analysis of antigen-specific T-cell responses both in peripheral blood lymphocytes and in tumor tissues has been previously validated (32). Primers for IFN- $\gamma$ , CD8, and TaqMan Probes (Custom Oligonucleotide Facility, Foster City, CA) were designed to span exon-exon junctions to prevent transcription of genomic DNA. To create a standard curve, the cDNA was generated by reverse transcription using the same technique used for the preparation of test cDNA. IFN- $\gamma$  and CD8 cDNA was amplified by PCR using the same primers designed for the real-time PCR, purified and quantified by UV spectrophotometry. The number of cDNA copies was calculated using the molecular weight of each gene amplicon. Serial dilutions of the amplified gene at known concentrations were tested by real-time PCR. Quantitative real-time PCR reactions of cDNA specimens, cDNA standards, and water as negative control were conducted in a total volume of 25  $\mu$ L with TaqMan Master mix (Perkin-Elmer), 400 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: IFN- $\gamma$  (forward) 5'-AGCTCTGCATCGTTTGGTTC, IFN- $\gamma$  (reverse) 5'-GTTCATATCCGCTACATCTGAA; IFN- $\gamma$  (probe) FAM-TCTTGCGTGTGATCGCAGGACCA-TAMRA; CD8 (forward) 5'-CCCTGAGCAATCCATCATG; CD8 (reverse) 5'-GTGGGCTTCGCTGGCA; and CD8 (probe) FAM-TCAGCACTGTCGCGCGCTTC-TAMRA. The thermal cycler variables were 2 min at  $50^{\circ}\text{C}$ , 10 min at  $95^{\circ}\text{C}$ , 40 cycles of  $95^{\circ}\text{C}$  for 15 s involving denaturation and  $60^{\circ}\text{C}$  for 1 min. Standard curve extrapolation of copy number was done for both IFN- $\gamma$  and CD8. The calculated number of copies of IFN- $\gamma$  mRNA in each

sample was normalized to the number of copies of CD8 mRNA by dividing the number of copies of IFN- $\gamma$  transcripts by the number of copies of CD8 transcripts. All PCR assays were done in duplicate and reported as the mean. A 2-fold difference in gene expression was found to be within the discrimination ability of the assay.

**Flow cytometric analysis.** PBMC were stimulated with peptide-loaded APC and stained for intracellular IFN- $\gamma$  production using BD Cytotex/Cytoperm Plus Kit (BD, Becton Dickinson) according to the manufacturer's instructions. Briefly, C1R-A2 cells were washed thrice in serum-free complete medium and incubated with test peptide at 10  $\mu$ g/mL for 2 h in complete medium at 37°C and 5% CO<sub>2</sub>. C1R-A2 cells were then washed once, irradiated (7,500 cGy) and suspended with  $1 \times 10^6$  PBMC at a 1:1 ratio in complete medium containing Golgi Stop. Cells were cocultured for 6 h at 37°C and 5% CO<sub>2</sub>. Unpulsed C1R-A2 cells and C1R-A2 cells pulsed with gp100 peptide (irrelevant peptide) were used as negative controls. Positive controls were done by stimulating PBMC with 5  $\mu$ g/mL of staphylococcus enterotoxin B (Sigma-Aldrich). T cells were then stained by incubation with monoclonal antibodies (BD) conjugated with allophycocyanin (CD3), and respectively, phycoerythrin or peridinin chlorophyll protein (PE or PerCP: CD8). The intracellular staining for IFN- $\gamma$  was done after fixation and permeabilization by using fluorescein isothiocyanate (FITC) and PE-conjugated monoclonal antibodies (BD), respectively. Data acquisition was done on FACSCalibur and was analyzed using CellQuest Software (BD).

For phenotypic characterization, PBMC were stimulated according to the protocol described above. CD8<sup>+</sup> T cells were stained by incubation with PerCP-conjugated monoclonal antibodies (BD). CD45RA expression was determined by using allophycocyanin-conjugated monoclonal antibodies (BD). IFN- $\gamma$  (PE) and CCR7 (FITC) monoclonal antibodies (BD) were added to determine antigen expression after fixation and permeabilization.

**Immunohistochemistry.** For the determination of survivin expression in multiple myeloma cells, 4- $\mu$ m sections of routinely processed, EDTA-decalcified and paraffin-embedded bone marrow trephine biopsies were stained using standard procedures. Briefly, immunohistochemical studies used an avidin-biotin peroxidase method with diaminobenzidine chromogen. After heat-induced antigen retrieval (microwave oven for 30 min at 250 W), immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ). Antibodies were applied to identify survivin (mouse monoclonal, clone 6E4, final dilution 1:200; Cell Signaling Technology, Beverly, MA), and the plasma cell marker CD138 (mouse monoclonal, clone MT15, final dilution 1:100; DAKO, Hamburg, Germany), which served to identify the extent and pattern of bone marrow infiltration. The dilutions had been established using adequate controls. Negative controls were obtained by omitting the primary antibodies. To quantitate the immunohistochemical expression of survivin, a scoring system similar to that of Lu et al. was used (36). The mean percentage of survivin-positive cells was estimated as <5% (0), 5% to 25% (1), 25% to 50% (2), 50% to 75% (3), and >75% (4). Staining intensity was classified as negative (0), weak (1), moderate (2), or intense (3). In order to identify neoplastic cells for evaluation of survivin expression, serial sections were stained with 116E and CD138. Only bone marrow trephines with an infiltration of plasma cells of >10% were evaluated. The percentage of positive tumor cells and staining intensity were multiplied to produce a final weighted score for each case. Cases with a weighted score of  $\leq 1$  were defined as negative; all others were defined as positive.

**Statistical analysis.** To determine specific response to stimulation, mRNA for IFN- $\gamma$  from PBMC stimulated with test peptide versus unpulsed APC (C1R-A2, background) was detected by quantitative PCR. The IFN- $\gamma$  mRNA copy number was first corrected for CD8 mRNA. A cutoff value of 2.0 for the ratio of IFN- $\gamma$  mRNA obtained from CD8<sup>+</sup> T cells stimulated with relevant test peptides to that obtained from PBMC stimulated with unpulsed APC was considered to be evidence of epitope specificity. The cutoff value was derived by analyzing IFN- $\gamma$  mRNA

transcripts detectable in PBMC both from healthy donors and patients stimulated with gp100 (209-2M; irrelevant peptide) to background. Analyses of these PBMC identified a mean ratio of 1.0 (range, 0.95-1.04) with 95% and 99% confidence intervals of 1.0  $\pm$  1.08 and 1.0  $\pm$  1.11, respectively, a SE of 0.04, and a SD of 0.2. The cutoff ratio (stimulation index, SI) was estimated by adding the mean to three SDs, which was equivalent to 1.6. To minimize the possibility of falsely considering CD8<sup>+</sup> T cells immunoreactive, we accepted a 2-fold increase in stimulated/unstimulated IFN- $\gamma$  transcript ratio as evidence of epitope-specific reactivity. Wilcoxon's rank sum test was calculated to determine whether there was a statistically significant difference in T-cell response to test peptides between normal healthy individuals and patients. Statistical significance was achieved at  $P < 0.05$ .

## Results

**Survivin-specific T cells can be detected in patients with multiple myeloma.** To determine whether CD8<sup>+</sup> T cells circulate in patients with multiple myeloma and normal healthy individuals, we analyzed T cells obtained directly from the peripheral blood of individuals without *in vitro* expansion. IFN- $\gamma$  mRNA production in peptide-stimulated T cells was determined using quantitative PCR. We analyzed 23 patients with multiple myeloma (16 males, 7 females; median age, 62 years) and 21 normal healthy individuals. Most of the patients were analyzed in advanced disease (stage III,  $n = 18$ ), the latter in stage II ( $n = 1$ ) and stage I ( $n = 4$ ). At the time of analysis, 8 patients showed a progressive disease and 15 patients a stable disease based on classical staging criteria. Clinical data are shown in Table 1. All analyzed individuals were positive for HLA-A2.1 allele. For positive controls, T cells were stimulated with staphylococcus enterotoxin B, negative controls were done using irrelevant peptide (gp100). A positive response was defined as  $\geq 100$  IFN- $\gamma$  mRNA copies/ $10^4$  CD8 copies and a SI of  $\geq 2$ , where SI = IFN- $\gamma$  mRNA copies/ $10^4$  CD8 copies in peptide-pulsed C1R-A2 cell cultures/unpulsed cultures. Analyzing T cell reactivity of individuals at least at two different time points showed a high degree of reproducibility.

Survivin-specific T-cell responses were detected in 1 of 21 (4.7%) healthy individuals (SI 7; Fig 1; Table 2). In contrast, 9 of 23 (39.1%) patients had a positive response to survivin antigen with a SI range from 2.9 to 83 ( $P = 0.0026$ ). The mean SI in the patients was 8.61 and 1.32 in normal individuals. None of the patients or healthy individuals had a positive response to gp100 (Table 2). One of the patients having a T-cell response to survivin antigen (patient 4) had an additional history of ovarian cancer in complete remission at the time of analysis. Five of nine (56%) patients having a positive T-cell response to survivin antigen showed progressive disease and four of nine (44%) patients showed stable disease. In contrast, patients without T-cell response to survivin antigen showed progressive disease in 3 of 14 patients (21%) and stable disease in 11 of 14 (79%) patients.

We additionally analyzed the T-cell reactivity to mucin 1 (MUC1), a glycosylated type I transmembrane glycoprotein that has recently been identified as a tumor-associated antigen on most myeloma cell lines, and that has been proposed as a candidate for peptide vaccination (29, 37). Neither patients nor healthy individuals showed a positive response to MUC1 peptides (Table 2). To confirm the specificity of survivin-reactive T cells detected by quantitative PCR, intracellular detection of IFN- $\gamma$  by flow cytometry was done. Peptide

**Table 1.** Patients and clinical properties

Patient no.	Sex	Age (y)	Stage*	Treatment	Years after diagnosis
1	M	66	IIIA, P	aHSCT	0
2	F	52	IIIA, P	ID	0
3	F	66	IIIA, P	aHSCT	4
4 <sup>†</sup>	F	66	IIa, S	None	3
5	F	57	IIIA, P	aHSCT, IFN	3
6	M	68	IIa, S	None	10
7	M	64	IIIB, P	CAD	0
8	M	64	IIIA, S	aHSCT, IFN, TCD	3
9	F	54	IIIA, P	aHSCT, TCD	3
10	M	62	IIIA, S	CAD	0
11	M	64	IIa, S	None	0
12	M	68	IIIA, S	aHSCT, IFN, TCD	2
13	M	69	IIIA, S	aHSCT, IRD, CAD, TCD	7
14	M	56	IIIB, S	aHSCT, IFN	1
15	M	67	IIa, P	aHSCT	0
16	F	54	IIa, S	0	4
17	M	61	IIIA, P	aHSCT	0
18	M	58	IIIB, S	ID	0
19	F	65	IIIA, S	aHSCT	0
20	M	61	IIIA, S	VAD, IEV	0
21	M	57	IIIA, S	ID	6
22	M	62	IIIA, S	aHSCT	0
23	M	70	IIIA, S	aHSCT, IFN	3

Abbreviations: None, not on active treatment at the time of analysis; aHSCT, autologous stem cell transplantation; TCD, thalidomide/darifenomycin/dexamethasone; VAD, vincristine/doxorubicin/dexamethasone; IEV, ifosfamide/epirubicin/etoposide; CAD, cyclophosphamide/adriamycin/dacarbazine; ID, idarubicin/dexamethasone; IFN, IFN- $\alpha$ ; IRD, idarubicin, ribomustin, and dexamethasone.

\*Salmon/Durie stage at time of analysis (P, progressive; S, stable, based on classical staging criteria).

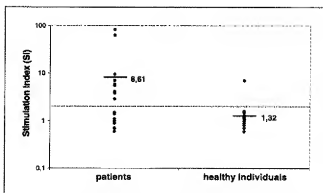
<sup>†</sup>Patient with multiple myeloma and additional history of ovarian cancer in complete remission at the time of analysis.

specificity determined by quantitative PCR was previously confirmed by independent studies (including our group) using intracellular cytokine assays showing strong correlation between both assays (31, 38, 39). In multiple experiments optimizing quantitative PCR assay conditions in our study, we compared IFN- $\gamma$  mRNA expression following cytomegalovirus pp65<sub>495-503</sub> peptide stimulation (in cytomegalovirus-positive individuals) with intracellular IFN- $\gamma$  production confirming strong correlation ( $R^2 = 0.97$ ; data not shown). A response was considered positive in intracellular cytokine assays if the percentage of IFN- $\gamma$ -producing T cells (CD3<sup>+</sup>, CD8<sup>+</sup> gated) cultured with peptide-pulsed APC was 2-fold or more higher compared with T cells cultured with unpulsed APC. Data from intracellular cytokine assays and corresponding data from quantitative PCR of three patients [patients 4 (A), 8 (B), and 15 (C)] with a positive response to survivin antigen are shown in Supplementary Fig. S1 and S2. IFN- $\gamma$  production of unpulsed APC was detected in 0.07%, 1.87%, and 0.05% of CD3<sup>+</sup>, CD8<sup>+</sup> gated cells (patients 4, 8, and 15), whereas a specific IFN- $\gamma$  production of peptide-pulsed APC was observed in 0.98%, 7.56%, and 0.46%, respectively. IFN- $\gamma$  production of gp100-peptide (negative control) was detected in 0.1%, 1.8%, and 0.03% of CD3<sup>+</sup>, CD8<sup>+</sup> gated cells, respectively.

Survivin-reactive T cells can be identified as terminally differentiated effector T-cells (CD8<sup>+</sup> CD45<sup>+</sup>, CCR7<sup>-</sup>). To further characterize survivin-reactive T cells, we determined the expression of CD45RA and CCR7 of specifically IFN- $\gamma$ -producing CD8<sup>+</sup> T cells. Figure 2 displays representative data from two patients [patients 4 (A) and 15 (B)] demonstrating that most of the T cells that specifically produced IFN- $\gamma$  after survivin peptide stimulation had the phenotype CD8<sup>+</sup>,

CD45RA<sup>+</sup>, CCR7<sup>-</sup>. T cells with this functional phenotype were recently described as terminally differentiated effector T cells exerting direct lytic activity (40, 41).

**Survivin expression in myeloma cells from patients after bone marrow trephine biopsies.** In order to determine survivin expression in myeloma cells, we analyzed formalin-fixed paraffin-embedded bone marrow trephine biopsies from patients with myeloma. Biopsies from 13 of 23 patients were



**Fig 1.** CD8<sup>+</sup> T-cell response to stimulation with HLA-A\*0201-restricted survivin peptide in patients with multiple myeloma and in healthy individuals. PBMC from patients with multiple myeloma ( $n = 23$ ) and healthy individuals ( $n = 21$ ) were incubated for 3 h with unpulsed antigen-presenting cells or APCs pulsed with survivin peptide. Points, SI of single experiments in which SI is determined by the ratio of IFN- $\gamma$  mRNA copy number obtained from PBMC stimulated with relevant test peptides to that obtained from PBMC stimulated with unpulsed APCs. A cutoff value of 2.0 (dotted line) was considered to be evidence of epitope specificity. Bars, mean SI for peptide stimulation.

**Table 2.** CD8<sup>+</sup> T cell reactivities to peptides in patients and healthy individuals

	SI*				SEB
	Survivin	gp100	MUC1a	MUC1b	
Patients <sup>†</sup>					
1	0.7	0.4	0.9	0.4	134
2	1	1.1	0.9	0.9	99
3	<b>4.1</b>	0.9	1.1	1	138
4	<b>63</b>	0.9	1.7	1.2	117
5	0.9	1.1	0.9	0.9	139
6	<b>0.7</b>	0.9	1	0.7	156
7	<b>5.8</b>	0.9	1.1	1.1	126
8	<b>83</b>	1.1	1.2	1.8	128
9	<b>9.5</b>	1.3	1.3	1.3	130
10	1	0.8	1	0.8	138
11	<b>2.9</b>	0.8	0.9	0.7	128
12	1.1	0.9	1	0.9	145
13	0.6	0.9	0.8	0.9	150
14	1.5	1	1	0.9	137
15	<b>3.8</b>	0.9	0.9	1	120
16	1.1	0.7	0.9	0.9	142
17	<b>5.5</b>	1	0.9	1	134
18	1.4	0.7	0.8	0.7	115
19	0.9	0.9	0.8	0.9	124
20	<b>7</b>	1.2	0.9	1	130
21	0.9	1.1	0.8	0.9	114
22	0.7	1.1	1	0.9	120
23	1.1	1.3	1.1	1.2	136
Healthy individuals <sup>†</sup>					
1	1.3	1.1	0.7	0.7	124
2	1.1	0.7	1.3	1.2	135
3	0.7	0.6	0.6	0.3	150
4	1	1	0.8	1.5	118
5	1.1	1.5	1.2	1	150
6	1.1	1.8	0.9	0.7	128
7	<b>7</b>	1.1	1.2	1.1	145
8	1	1.1	1.4	1.2	161
9	1.2	0.7	1.2	0.7	110
10	1.5	1.3	1.5	0.8	116
11	0.6	0.5	0.9	0.6	134
12	0.8	0.8	0.8	0.7	119
13	1.5	1.3	1.8	1.5	131
14	1	1	1.1	1.1	129
15	1.2	1.3	1.2	1.5	137
16	0.8	0.7	0.8	1	111
17	0.9	0.9	0.9	0.7	135
18	0.6	0.9	1.5	1.3	116
19	1.6	1.7	1.2	1.5	126
20	1.1	1.3	1.2	1.1	116
21	0.7	0.7	0.8	0.9	136

\*SI determined by the ratio of IFN- $\gamma$  mRNA copy number obtained from PBMC stimulated with relevant test peptides to that obtained from CD8<sup>+</sup> T-cells stimulated with unpulsed antigen-presenting cells. A cutoff value of 2.0 was considered to be evidence of epitope specificity. SI displayed in boldface represent positive reactivities.

†Staphylococcus enterotoxin B used as positive control.

‡Corresponding numbers of patients/healthy individuals were maintained throughout this article.

available for analysis. Diagnosis was based on standard clinical and histopathologic criteria. It was possible to assess 11 of 13 bone marrow trephines for the immunohistochemical expression of survivin (Table 3). Two biopsies showed a plasma cell infiltrate of  $\leq 10\%$  for all cells and were therefore omitted. The remaining trephines showed a clearly recognizable infiltration by neoplastic plasma cells (mean, 61%; range, 15-90%) as

proven by conventional H&E staining and additional immunohistochemical detection by CD138. Based on the weighted scores, survivin expression in myeloma cells was detected in 7 of 11 (63.6%) cases of multiple myeloma (survivin expression in myeloma cells of patient 9; Fig 3). Survivin signals were predominantly localized in the nucleus. In two of the seven patients (patients 9 and 15), a survivin-specific T-cell response was detected in the peripheral blood (Tables 2 and 3). In one patient having a survivin-specific T-cell response, the bone marrow biopsy showed a plasma cell infiltrate of  $\leq 10\%$  and had to be omitted. Analyzing peritumoral T-cell infiltration in bone marrow trephines from patients 9 and 15 by immunostaining of CD3<sup>+</sup> cells showed a T-cell infiltration of 5% to 10% (patient 9) and 10% to 30% (patient 15), respectively (data not shown). Three of seven (42%) patients with survivin expression in myeloma cells showed progressive disease and four of seven (58%) patients showed stable disease.

Analyzing patients with no detectable survivin expression in myeloma cells showed progressive disease in one of four individuals (25%) and stable disease in three of four (75%) individuals.

## Discussion

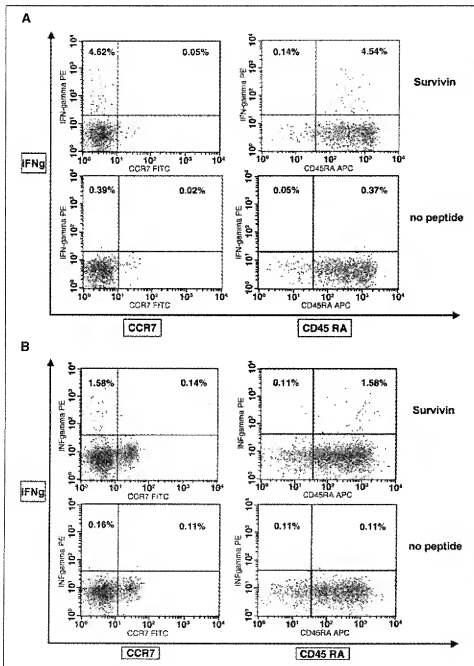
Survivin is a member of the inhibitor of apoptosis gene family that has recently been suggested as a promising target antigen in different malignancies (8, 9, 11, 42, 43). Both spontaneous specific T-cell reactivity and antibody response to survivin have been recently detected in patients with leukemia, melanoma, breast, lung, and colorectal cancer (15-20). It has been shown that survivin plays a critical role in the survival and proliferation of human myeloma cells (22, 23), and that *in vitro*-induced survivin-specific cytotoxic T cells from healthy individuals have the ability to lyse multiple myeloma cell lines (16). To our knowledge, no other groups have thus far studied the T cell-mediated immunity to survivin in patients with myeloma. This study provides, for the first time, direct evidence that CD8<sup>+</sup> T cells recognizing survivin antigen circulate in patients with multiple myeloma. Using quantitative PCR, we found that CD8<sup>+</sup> T-cells reactive to a recently described HLA-A2-restricted survivin peptide are present in 39% of the patients and in only 4.7% of the healthy controls, suggesting that specific T-cell responses were induced in response to myeloma cells. Our data are in concordance with a recent study in which spontaneous T-cell reactivities to survivin were almost completely restricted to patients with cancer (18). To investigate whether there is a relationship between survivin-specific immune response and active disease, we analyzed the clinical course of patients and found that those with a T-cell response to survivin antigen more often showed a progressive than stable disease (56% versus 44%) compared with patients without a T-cell response who, for the most part, showed stable disease (79%). Although data are not significant and further studies analyzing a larger number of patients are required, it is suggested that T-cell reactivity is elicited in response to tumor cells (to immunologically counteract tumor progression) which could be due to increased antigen expression by tumor cells or to increased antigen presentation by antigen-presenting cells in the bone marrow. The analysis of two patients having survivin expression in myeloma cells and T-cell response to survivin antigen in the peripheral blood showed T-cell infiltration

(up to 30%) in bone marrow areas affected by myeloma cells, which supports the hypothesis that specific T-cell stimulation occurs at the location of the tumor. Survivin is highly expressed in most human cancer cells of epithelial and hematopoietic origin and overexpression is associated with cancer progression, poor prognosis, resistance, and short patient survival (8, 9, 13, 14). It is supposed that the overexpression of survivin in the cytoplasm of tumor cells and rapid degradation by the proteasome-related mechanism results in an increased expression of survivin-derived epitopes on the surface of tumor cells leading to the induction of a specific T-cell response.

The cytolytic abilities of survivin-reactive T-cells detected by measuring specific IFN- $\gamma$  production cannot directly be assessed

*ex vivo*. To provide more insight into that question, we characterized the differentiation phenotype of survivin-reactive CD8<sup>+</sup> T-cells. Survivin-reactive CD8<sup>+</sup> T-cells displayed the phenotype CD45RA<sup>+</sup>, CCR7<sup>-</sup>, corresponding to terminally differentiated effector T-cells. It has recently been shown that these cells have the highest cytotoxic potential and directly mediate tumor cytotoxicity (40, 41). However, long-term follow-up analysis to determine the effect of survivin-specific T-cell response on the course of disease is needed.

Because survivin-specific T-cell responses were mostly restricted to the patients, we did immunohistochemical analyses of patients' bone marrow biopsies to determine the survivin expression in tumor cells. Potential immunotherapeutic



**Fig. 2.** Flow cytometric analysis of the differentiation phenotype of CD8<sup>+</sup> T-cells specifically producing IFN- $\gamma$  in response to survivin in patients with multiple myeloma. Analysis was done by four-color flow cytometry in patient 4 (A) and patient 15 (B). The IFN- $\gamma$ /CCR7 and IFN- $\gamma$ /CD45RA profiles of CD8<sup>+</sup> T-cells after cocultivation with unpulsed (no peptide, bottom) and survivin peptide-pulsed antigen-presenting cells (survivin, top). Frequencies of T-cells that produced IFN- $\gamma$  are shown in each quadrant as a percentage of gated CD8<sup>+</sup> T-cells. PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanin.

**Table 3.** Immunohistochemical analysis of survivin expression in bone marrow trephines of patients with multiple myeloma

Patient no.*	Plasma cell infiltration (%) <sup>1</sup>	Mean percentage <sup>2</sup>	Staining intensity <sup>3</sup>	Final scoring <sup>4</sup>
2	20	1	3	3
5	40	0	1	1
6	15	3	2	6
9†	70	2	3	6
10	90	1	3	3
11†	5	n.a.	n.a.	n.a.
12	10	n.a.	n.a.	n.a.
13	80	3	1	3
14	90	0	0	0
15†	90	1	3	3
18	60	0	0	0
21	90	0	1	1
22	30	3	3	9

Abbreviation: n.a., not analyzed due to low plasma cell infiltration (<10%).

\*Corresponding numbers of patients and healthy individuals were maintained throughout this article.

<sup>1</sup>Percentage of plasma cells out of total counted cells in bone marrow trephines.

<sup>2</sup>Mean percentage of survivin expressing plasma cells estimated as <5% (0), 5% to 25% (1), 25% to 50% (2), 50% to 75% (3), and >75% (4).

<sup>3</sup>Intensity of immunohistochemical survivin staining classified as negative (0), weak (1), moderate (2), or intense (3).

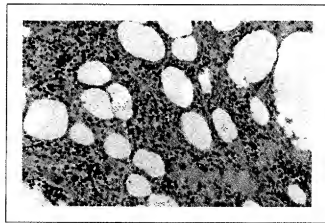
<sup>4</sup>The percentage of survivin expressing plasma cells and staining intensity were multiplied to produce a final weighted score for each case. Cases with weighted score ≤1 were defined as negative, all others were defined as positive.

†Patients with a survivin-specific T cell response.

strategies using survivin as a target antigen requires its expression in tumor cells. Survivin expression was detected in myeloma cells in almost 64% of the patients analyzed. This is in accordance with recent studies in which survivin expression was found in tumor cells from ~60% of patients with diffuse large B-cell lymphoma, acute myelogenous, and lymphocytic leukemia (44–46). Not all patients showing a positive survivin expression had a specific T-cell response to survivin. This might be due to the affinity of survivin-specific T cells or to quantitative differences in the presentation of survivin-derived epitopes by myeloma cells. Further studies analyzing specific T-cell affinity and performing quantitative analysis of survivin expression by real-time PCR would be helpful to determine whether these are causally connected to the level of T-cell response. Because survivin expression in other malignancies is associated with disease progression and poor prognosis, we investigated whether there was a relationship between survivin expression in myeloma cells and active disease. Forty-two percent of patients with survivin expression in myeloma cells showed progressive disease compared with only 25% of patients with no survivin expression. This suggests that positive survivin expression might be correlated with disease activity. However, data are not significant and further studies analyzing a larger number of patients are required.

It has been reported that survivin is expressed in thymic cells, bone marrow–derived hematopoietic progenitor cells, colonic

epithelial cells, and activated epithelial cells (12). One might raise the hypothesis that autoimmune reactions will be induced after performing immunotherapy (either adoptive or by vaccination) using survivin as a target antigen. The patients in our study, having a specific T-cell response to survivin, did not show clinical signs of gastrointestinal disease or vascular involvement. Some of the patients had decreased levels of hemoglobin and/or white blood count, but due to the biology of multiple myeloma affecting hematopoiesis and due to potential therapy effects, it might be difficult to distinguish between the effects caused by survivin-reactive T-cells or by disease respectively therapy. However, a vaccination study that was recently done in patients with melanoma in which survivin-specific T-cells could be induced by survivin peptide–pulsed dendritic cells did not show any clinical or histochemical changes (21). We additionally tested T cell reactivity to MUC1, a glycosylated type I transmembrane glycoprotein that has been identified as a tumor-associated antigen on most myeloma cell lines and that has been proposed as a candidate antigen for vaccination treatments (29). In contrast to recent studies in which T cells recognizing MUC1-derived peptide epitopes have been found in the peripheral blood of healthy individuals (29, 37) or in the peripheral blood and/or bone marrow of patients with myeloma (47, 48), we could not detect any response in our patients or healthy individuals. In the studies mentioned, MUC1-specific T-cells were mainly detected after *in vitro* T-cell expansion, suggesting that the MUC1-specific T-cells circulate in very low frequencies. We used a highly sensitive assay to detect spontaneous antigen-specific T-cells; however, in the individuals tested, MUC1-specific T-cells might be circulating in frequencies below the detection limit of the assay or might even be absent. Further studies using *in vitro* T-cell expansion or analyzing patients' bone marrow could be helpful to clarify the existence of MUC1-specific T-cells in the patients analyzed.



**Fig 3.** Immunohistochemical analysis of survivin expression in the bone marrow of a patient with multiple myeloma. Immunohistochemical staining of bone marrow was done using an avidin-biotin peroxidase method with diaminobenzidine chromogen. To quantitate the immunohistochemical expression of survivin, a scoring system similar to that of Lu et al. was used (36). The mean percentage of survivin-positive cells was estimated as 45%, 5% to 25%, 25% to 50%, 50% to 75%, and >75%. Staining intensity was classified as negative, weak, moderate, or intense. In order to identify neoplastic cells for evaluation of survivin expression, serial sections were stained with H&E and CD138. Only bone marrow trephines with an infiltration of plasma cells of >10% were evaluated. Survivin expression in 25% to 50% of myeloma cells with an intense staining pattern (brown; magnification, ×200).

In conclusion, we provide for the first time, evidence for T-cell reactivity against survivin antigen in patients with multiple myeloma. Our data suggest the immunogenicity of survivin antigen in multiple myeloma and that survivin could serve as useful target antigen for T cell-based immunotherapeutic strategies in the treatment of multiple myeloma. Because the bone marrow was recently identified as being important for tumor surveillance, containing a pool of antigen-specific T cells (49), and because multiple myeloma represents a disease that

is mainly located in the bone marrow, we plan further investigations analyzing survivin-specific T-cell reactivities in patients' bone marrow to get more information about the relevance of that compartment in controlling tumor cells.

## Acknowledgments

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## **Appendix B**

Brief Communication

**A survivin specific T-cell clone from a breast cancer patient display universal tumor cell lysis**

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**Running title:** A survivin specific T-cell clone

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## **Abstract**

Survivin is an attractive candidate for cancer immunotherapy since it is over expressed in most common human cancers, poorly expressed in most normal adult tissues and is essential for cancer cell survival. Previously, we and others have demonstrated that survivin-specific immune responses are present in cancer patients. However, a significant limitation of these findings has been that antigen-specific lysis of tumors was achieved using polyclonal T-cell lines rather than a specific T cell clone. In the present study we isolated and expanded survivin specific cytotoxic T lymphocyte (CTL) clones from the peripheral blood of cancer patients. The survivin specific CTL clones efficiently lysed a large panel of tumor cells of different origin, i.e. breast cancer, colon cancer, and melanoma cells. The data support the notion that survivin may serve as a universal target antigen for anti-cancer immunotherapy.

## **Introduction**

Survivin is abundantly and ubiquitously present in development, undetectable in most adult tissues, and prominently re-expressed in virtually every human cancer. Thus, one of the most significant features of survivin is its preferential expression in tumor versus normal tissues <sup>1,2</sup>. Thus, survivin is over-expressed in almost all cancers including lung, colon, breast, pancreas, stomach, liver, ovary and prostate cancer, as well as melanoma, and hematopoietic malignancies <sup>3-5</sup>. Survivin has attracted attention as a unique member of the inhibitor of apoptosis (IAP) gene family with a potential dual role in apoptosis inhibition and regulation of mitosis <sup>2</sup>. Accordingly, down regulation of survivin would severely inflict the survival capacity of tumor cells, which highlights this protein as a prime target candidate for therapeutic vaccinations against cancer, since it is not subject to immune selection. Hence, the universal expression by the vast majority of tumors as well as its natural functional involvement in oncogenic transformation makes survivin an attractive tumor antigen. In

this regard, several recent reports have showed that survivin is among the tumor antigens that serve as targets for immune-mediated tumor destruction in cancer patients <sup>6</sup>. In line of this, in the present study we isolated and expanded a survivin specific T-cell clone from a melanoma patient and examined its ability to kill a large panel of different cancer cells.

## **Materials and Methods**

Peripheral blood was attained from a HLA-A2 positive breast cancer patient. Patient characteristics: Age 51 years, disseminated disease in lung, liver, lymph nodes, and skin from a ductal carcinoma, estrogen and progesterone receptor negative. Prior treatment for metastatic disease included four lines of chemotherapy (epirubicin, docetaxel, capecitabine, and vinorelbine), no chemotherapy were given within 4 weeks prior to blood sampling. Peripheral blood lymphocytes (PBL) were isolated using lymphoprep separation. PBL were analyzed by flow cytometry using FACSaria. T cells were stained with an HLA-A2/Sur1M2 (LMLGEFLKL) Pro5 pentamer followed by staining with the fluorochrome-coupled CD8 antibody. Double positive cells were sorted for cloning. The cells were sorted as single cells into 96 well plates containing a mixture of irradiated PBL from three healthy donors in X-vivo with 5% heat-inactivated human serum, 1 ug/ml PHA and 120 U/ml IL-2. The cells were incubated at 37C/5% CO<sub>2</sub>. After expansion using IL-2 and irradiated PBL mix, the specificity and functional capacity of the growing clones was analyzed in standard <sup>51</sup>Cr release assays. To this end, either T2 cells without peptide or loaded with Sur1M2 (10μM) served as targets as well as different cancer cell lines: Breast cancer (MDA-MB-231, CAMA-1 and ZR-1-75), colon cancer (HCT-116) and melanoma cancer (FM9, FM92, ESTDAB-001, 006, 007, 014, 017, 019, 021, 026, 027, 028, 029, 033, 100, and 117).

## Results and Discussion

Spontaneous anti-survivin T-cell reactivity has been described in cancer patients suffering from a huge range of cancers of different origin, e.g. breast and colon cancer, lymphoma, leukaemia and melanoma<sup>7</sup>. To explore the lytic capacity of survivin specific T cells we and others has previously showed that survivin-reactive T cell from bulk cultures were capable of lysing HLA-matched tumor cells of different tissue origin, including breast cancer cells, leukemia and melanoma<sup>7,8</sup>. However, a significant limitation of these findings was that antigen-specific, MHC-restricted lysis of tumors was achieved using polyclonal T cell lines rather than a specific T cell clone. Previously, we have identified a low affinity HLA-A2-restricted survivin derived peptide epitope, Sur1, (position 96-104; LTLGEFLKL)<sup>9</sup>. The weak binding affinity of Sur1 to HLA-A2 was improved by replacing threonine at position 2 with a better anchor residue (methionine; Sur1M2). This measure enabled the construction of stable HLA-A2/peptide pentamer complexes<sup>10</sup>. Hence, to further characterize the universal character of survivin, HLA-A2/Sur1M2 pentamer positive CD8 cells were sorted by FACS from PBL from a breast cancer patient. The cells were sorted as single cells into 96 well plates containing a mixture of irradiated PBL from three healthy donors, PHA and IL-2. After expansion, the specificity of the growing clones was analyzed in standard <sup>51</sup>Cr release assays. To this end, either T2 cells without peptide or loaded with Sur1M2 (10 $\mu$ M) served as targets. A number of clones exclusively killed T2 cells pulsed with Sur1M2 (one of these depicted in Figure 1A). This Sur1M2 specific CTL clone was further used to test the capacity to kill cancer cell lines of different origin. The HLA-A2 positive breast cancer cell lines CAMA-1 and MDA-MB-231, the HLA-A2 positive colon cancer cell line HCT-116, and the HLA-A2 negative breast cancer cell line ZR-1-75 were used as target cells. The Sur1M2 specific CTL clones efficiently lysed all HLA-A2 positive cells, whereas in contrast, no cytotoxicity was observed against the HLA-A2 negative breast cancer cell line ZR-1-75 (Figure 1B). Furthermore, a large panel of melanoma cell lines was examined; the

CTL clone effectively lysed all the melanoma cell lines except one cell line (Figure 1B). All examined cell lines expressed Survivin RNA (Fig. 1B). The expression of Survivin were obtained from the ESTDAB database (<http://www.ebi.ac.uk/ipd/estdab>). The Sur1M2 specific CTL clone effectively lysed ESTDAB-007 melanoma cells in a HLA-dependant matter as lysis could be completely blocked by incubation of target cells with antibody (Fig. 1A and B). Likewise, to show the HLA-A2/peptide restriction we used cold target inhibition. Hence, the addition of cold (unlabeled) T2 cells pulsed with the specific Sur1m2 peptide completely abrogated the killing of ESTDAB-007 melanoma cells, whereas the addition of T2 cells without peptide only showed a showed a reduced lysis of ESTDAB-007 (Fig. 1A and B). This dilution effect is occurring because the addition of 20 times as many target cells to the effectors completely changes the actual E:T ratio.

We did have any autologous cancer cells available, however, lysis of autologous tumor by Sur1M2 polyclonal T cells has been described previously <sup>11</sup>. All in all our data underline the universal character of the survivin and highlight survivin as an attractive target antigen for cancer immunotherapy. Being a universal tumor antigen, survivin may serve as a widely applicable target for anti-cancer immunotherapy.

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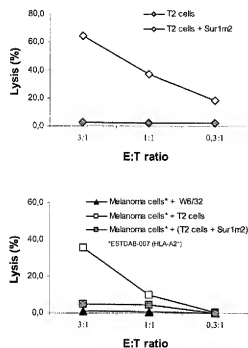
## Figure legends

### Figure 1:

(A) Peptide specificity and HLA-restriction of a Sur1m2 specific T-cell clone assayed by  $^{51}\text{Cr}$ -release assay: Lysis of T2 cells with no peptide or pulsed with Sur1M2 peptide, and specific lysis of the HLA-A2 positive melanoma cell line ESTDAB-007 without or with the addition of the HLA-class I specific antibody W6/32. In addition, unlabeled T2 cells either with or without the Sur1M2 peptide were added to ESTDAB-007 cells at a ratio of inhibitor to target cells of 20:1. (B) Functional capacity of a Sur1M2 specific T-cell clone to kill different cancer cell lines (melanoma, breast and colon cancer) assayed by  $^{51}\text{Cr}$ -release assay. All assays were performed in E:T ratio = 1:1. In addition, expression of Survivin RNA in the cancer cell lines is given (data obtained from the ESTDAB database (<http://www.ebi.ac.uk/ipd/estdab>)).

Figure 1

(A) Peptide specificity and HLA-restriction



(B): Lysis of cancer cells (% lysis)

Melanoma cancer	% lysis	Survivin expression*
ESTDAB-001	49%	++
ESTDAB-006	42%	+++
ESTDAB-007	36%	+
ESTDAB-014	23%	++
ESTDAB-017	20%	++
ESTDAB-019	45%	+++
ESTDAB-021	47%	++
ESTDAB-026	73%	+++
ESTDAB-027	45%	++
ESTDAB-028	-	+++
ESTDAB-029	61%	+++
ESTDAB-033	47%	++
ESTDAB-100	42%	+++
ESTDAB-117	35%	++
FM62	50%	ND
FM9**	-	ND

Breast cancer	% lysis	Survivin expression*
CAMA-1	26%	ND
MDA-MB-231	89%	ND
ZR-75**	-	ND

Colon cancer	% lysis	Survivin expression*
HCT-116	27%	ND

\*Data obtained from the ESTDAB-database (<http://www.ebi.ac.uk/ind/estdab>)

\*\*HLA-A2 negative

## **Appendix C**

## Vaccination of multiple myeloma patients with idiotypic-pulsed dendritic cells: immunological and clinical aspects

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**Summary.** Multiple myeloma (MM) is characterized by a clonal proliferation of malignant plasma cells in the bone marrow secreting a monoclonal immunoglobulin (paraprotein) with specific antigenic determinants, the idiotype (Id), which can be regarded as a tumour-associated antigen (TAA). In order to analyse the impact of a dendritic cell (DC)-based vaccine, 11 patients with advanced MM were treated with CD34 stem cell-derived dendritic cells that were pulsed with Id peptides. Subsequently, the patients received three boost immunizations every other week with a combination of Id and granulocyte-macrophage colony-stimulating factor (GM-CSF) (nine patients) or with Id peptide-pulsed dendritic cells again (two patients). The treatment was well tolerated with no side-effects. The present clinical study was a proof of concept analysis of dendritic cell-based vaccines in MM. The capacity of the dendritic cells to activate idiotypic-specific T cells was verified by *in vitro* stimulation experiments before the vaccination therapy. Immunological effects of the Id vaccination were

analysed by monitoring changes in anti-idiotypic antibody titres and idiotypic-specific T-cell activity. After vaccination, three out of 10 analysed patients showed increased anti-idiotypic antibody serum titres, indicating the induction of an idiotypic-specific humoral immune response. The idiotypic-specific T-cell response analysed by ELISpot was increased in four out of 10 analysed patients after vaccination, and one patient had a decreased plasma cell infiltration in the bone marrow. In conclusion, five out of 11 patients showed a biological response after vaccination. Thus, our data indicate that immunotherapy with Id-pulsed DCs in MM patients is feasible and safe. DC generated from CD34<sup>+</sup> progenitor cells can serve as a natural adjuvant for the induction of clinically relevant humoral and cellular idiotypic-specific immune responses in patients suffering from advanced MM.

**Keywords:** dendritic cells, CD34<sup>+</sup> progenitor cells, vaccination, idiotypic, multiple myeloma.

Multiple myeloma (MM) is a malignant clonal B-cell neoplasm with a poor prognosis and a mean survival of 1–3 years. Conventional chemotherapies, such as melphalan (MP), VAD (vincristine, adriamycin, dexamethasone) or related regimens, have shown no curative perspectives, underlining the need for immunotherapeutic approaches.

The malignant myeloma cells, mainly localized in the patients' bone marrow, secrete a monoclonal immunoglobulin (Ig), which can be found in the serum and urine (Mellstedt *et al.* 1984). These monoclonal immunoglobulins have somatically mutated variable regions and represent a

unique antigenic entity known as the idiotype (Id) (Vescio *et al.* 1995). Idotypic structures can be expressed and presented in a major histocompatibility complex (MHC)-restricted pattern on the cell surface of the myeloma plasma cells, thus serving as a highly specific tumour-associated antigen (TAA). The MHC-restricted presentation of Id determinants enables the plasma cell to serve as a target and antigen-presenting cell (APC) for idiotypic-specific T-cells (Weiss & Bogen, 1989; Yi *et al.* 1997a).

Dendritic cells (DCs) are professional antigen-presenting cells that are considered to be a suitable tool for vaccination therapy in tumours. In a variety of lymphoid and solid tumour models, DCs have proved their important function in the immunosurveillance of tumours. Until recently, the clinical application of DCs has been hampered by limited cell

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**Table 1.** Patient characteristics

Patient	Sex	Age	Clinical stage	Puraprotein	Prior therapies*
01	M	64	III	IgG/ $\kappa$	2 $\times$ MP, 6 $\times$ VAD, 6 $\times$ ID
02	F	75	II	IgG/ $\kappa$	25 $\times$ MP, 2 $\times$ ID
03	M	59	III	$\lambda$	2 $\times$ MP, 2 $\times$ VAD
04	F	68	III	IgG/ $\kappa$	20 $\times$ VCMF
05	M	61	III	IgA/ $\kappa$	17 $\times$ MP, 2 $\times$ VCAP, 2 $\times$ ID
07	M	64	III	IgG/ $\lambda$	3 $\times$ MP
08	F	67	III	IgG/ $\lambda$	11 $\times$ MP
09	F	42	III	IgA/ $\lambda$	1 $\times$ M, autologous stem cell transplantation
10	F	67	III	$\kappa$	3 $\times$ MP, 5 $\times$ D, 6 $\times$ VAD, 1 $\times$ M, autologous stem cell transplantation, R
11	M	57	III	IgG/ $\kappa$	14 $\times$ VAD, R
12	F	67	III	IgA/ $\kappa$	12 $\times$ VAD

\*M, melphalan; D, dexamethasone; MP, melphalan/prednisone; VAD, vincristine/adriamycin/dexamethasone; ID, idarubicin/dexamethasone; VCMF, vincristine/cyclophosphamide/alkalan/prednisone; VCAP, vincristine/cyclophosphamide/adriamycin/prednisone; R, roferon (IFN $\alpha$ ).

numbers and labour-intensive isolation procedures. Several groups have demonstrated that *in vitro* differentiation of peripheral blood monocytes or CD34<sup>+</sup> stem cells in the presence of defined cytokine cocktails, such as granulocyte-macrophage colony-stimulating factor/interleukin 4 (GM-CSF/IL-4) or GM-CSF/tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), resulted in functional, highly active APCs (Peters *et al.* 1987, 1991; Caux *et al.* 1992, 1996). Thus, sufficient DC numbers can be generated from the peripheral blood of cancer patients for immunotherapeutic approaches.

Indeed, recent studies in mice and humans with either primary or *in vitro* differentiated DCs as a natural adjuvant showed the induction of idiotypic-specific T-cell and B-cell responses (Pflamand *et al.* 1994; Bohlen *et al.* 1996; Hsu *et al.* 1996). Idiotypic-specific T cells have been detected in the peripheral blood of patients with MM (Yi & Osterborg, 1996; Yi *et al.* 1997b). However, they seem to be inefficient in the prevention of tumour growth. Although myeloma cells seem to be capable of presenting Id peptides, this does not lead to an effective antitumour response. However, effective Id presentation by professional APCs could probably evoke protective T- and B-cell immune

responses. In this context, DCs play a major role in the recruitment and activation of antigen-specific T cells (Inaba & Steinman, 1986; Inaba *et al.* 1987). This is mainly a result of their ability to present antigens effectively and to provide co-stimulation via CD80, CD86, CD40 and adhesion molecules CD11a, CD11c, LFA-3 and ICAM-1 (Egner *et al.* 1992; Hart *et al.* 1995; Inaba *et al.* 1995). Furthermore, immunoregulatory cytokines, such as IL-12, secreted by DCs promote the development of Th1 T cells (Ellis *et al.* 1991; Macatonia *et al.* 1993, 1995), which play a major role in tumour-specific T-cell responses in mouse and man (Zitvogel *et al.* 1996; Osterborg *et al.* 1998).

Most TAAAs do not elicit a tumour-specific immune reaction *in vivo*. This observation may result partially from reduced numbers of DCs or the lack of functional DCs at the tumour site. As described by Caux *et al.* (1995, 1996, 1997), tumour-infiltrating DCs from colon carcinoma and basal-cell skin tumours were found to lack important co-stimulatory molecules. Such deficiencies might be overcome by the administration of functional *ex vivo*-generated TAA-pulsed DCs.

**Table 2.** Therapy-related immunological and clinical effects.

Patient	No. of DCs	Toxicity grade	Increase in anti-idiotypic antibodies	Increase in idiotypic-specific T cells	Restaging
01	1 $\times$ 10 <sup>6</sup>	I	IgM + IgG	+	PD
02	1 $\times$ 10 <sup>6</sup>	I	—	—	PD
03	1 $\times$ 10 <sup>7</sup>	I	IgM + IgG	+	PD
04	1 $\times$ 10 <sup>7</sup>	I	—	—	PD
05	1 $\times$ 10 <sup>7</sup>	I	—	—	PD
07	2 $\times$ 10 <sup>7</sup>	I	—	—	PD
08	1 $\times$ 10 <sup>7</sup>	I	—	—	PD
09	2.5 $\times$ 10 <sup>6</sup>	I	—	—	PD
10	1 $\times$ 10 <sup>6</sup>	I	IgM	NE	SD*
11	4 $\times$ (2 $\times$ 10 <sup>7</sup> )	I	—	CD8	PD
12	4 $\times$ (2 $\times$ 10 <sup>7</sup> )	I	NE	CD4	PD

\*Minor response (decrease in plasma cells in the bone marrow).  
NE, not evaluated.

In this vaccination study, *in vitro*-differentiated functional DCs were used to induce idiotype-specific responses in patients with MM. Here, we report on the clinical and immunological effects of this Id vaccination approach.

## PATIENTS AND METHODS

**Patients.** Patients with biopsy-confirmed diagnosis of MM were staged according to Durie & Salmon (1975) (Table 1). The serum Id concentration [ $\text{IgG } \kappa$ ,  $\text{IgG } \lambda$ ,  $\text{IgA } \kappa$ ,  $\lambda$  light chain,  $\kappa$  light chain] ranged from 2 g/l to 50 g/l. Inclusion criteria consisted of a Karnofsky index of at least 60%, an expected life span of more than 6 months and a chemotherapy-free interval of at least 2 months. Patients with leucopenia ( $< 1.5 \times 10^9$  cells/l), lymphopenia ( $< 0.2 \times 10^9$  cells/l), known infection with hepatitis immunodeficiency virus (HIV), hepatitis A, B or C virus (HAV, HBV, HCV), cytomegalovirus (CMV), pregnancy, failure of immune reaction to recall antigens or acute infections were excluded. Written informed consent was given by each patient, and the study was approved by the local ethics committee according to the Declaration of Helsinki.

**Assessment of toxicity.** Therapeutic side-effects were evaluated according to the National Cancer Institute (NCI) toxicity criteria as grade I (asymptomatic, well tolerated), II (mild, tolerable), III (moderate, poorly tolerated) or IV (severe, life threatening).

**Evaluation of clinical response.** Before vaccination therapy, clinical examination of the patients, routine blood analysis and immunoelectrophoresis from the serum or urine were performed. Furthermore, the patients underwent X-ray examinations of the extremities, cranium, vertebral column and pelvis. A bone marrow biopsy immediately before and 3 months after vaccination was taken from patients 01 and 10. The clinical status and laboratory parameters were evaluated during the vaccination period every other week and at least until 150 d after vaccination. The restaging regarding the X-ray examination of osteolytic lesions was done 3 months after vaccination for all patients.

Complete remission (CR) was defined as the normalization of paraprotein concentration in the serum or urine and a decrease in plasma cells in the bone marrow to normal values. Partial remission (PR) was defined as a decrease of at least 50% in plasma cells in the bone marrow. Progressive disease (PD) was defined as an increase in plasma cells, increased paraprotein concentration or new osteolytic lesions, and stable disease (SD) was defined when the criteria of CR, PR or PD were not seen. Biological responses were defined as a reduction in the paraprotein or paraprotein components, the induction of anti-idiotypic antibodies or the detection of idiotype-specific T cells in peripheral blood.

**Isolation of idiotype.** Idiotype IgG was purified from the patients' sera and urine before vaccination therapy by affinity chromatography on a protein G column (Pharmacia Biotech, Uppsala, Sweden) using a high-performance liquid chromatography (HPLC) apparatus (BioCAD; Perspective Biosystems, Wiesbaden, Germany), whereas light chains

and IgA were isolated by size-exclusion chromatography. The eluates were immediately dialysed into acetate buffer (pH 4.0) and adjusted to a concentration of 1 mg/ml. IgG cleavage was attained by the addition of pepsin (25  $\mu\text{g}/\text{ml}$ ) (Boehringer Mannheim, Mannheim, Germany) and incubation for 24 h in a 37°C water bath. The reaction was terminated by the addition of 1:10 (v/v) 3.0 mol/l Tris base (Sigma, Deisenhofen, Germany), and the proteins were concentrated by centrifugation (20 min at 2000 g) on Centrprep membranes (Amicon, Witten, Germany) with a molecular weight cut-off of 30 kDa.  $\text{F(ab')}_2$  fragments were isolated by size-exclusion chromatography on a phenyl-sepharose column (HiLoad 26/60 Superdex 200; Pharmacia Biotech) equilibrated in 0.9% NaCl (Delta Pharma, Pfaffingen, Germany). The proteins were adjusted to a concentration of 200  $\mu\text{g}/\text{ml}$  in 0.9% NaCl, sterile filtered and stored in glass vials. Cleavage of  $\text{F(ab')}_2$  fragments into peptides was performed in 0.9% NaCl using proteinase K single-use columns (MöBitec, Göttingen, Germany). The  $\text{F(ab')}_2$  fragments and peptides were analysed by SDS-PAGE, anti-human heavy-chain and anti-human light-chain enzyme-linked immunosorbent assay (ELISA), size-exclusion chromatography and reverse-phase chromatography. Samples to be used for Id pulsing of DCs or for T-cell stimulation assays were tested for endotoxins by the Limulus assay (QCL-1000; BioWhittaker, Walkersville, MD, USA), as well as for bacterial and viral contamination. Specimens containing more than 5 IU/ml endotoxin were not used in the T-cell stimulation assays or for clinical application.

**Generation of dendritic cells.** DCs were differentiated from  $\text{CD34}^+$  peripheral blood progenitor cells, which were obtained from leukapheresis material.  $\text{CD34}^+$  cells were mobilized for 5 d with G-CSF (Granocyte, 450  $\mu\text{g}$ ; Essex Pharma, Munich, Germany). The volume of leukapheresis products ranged from 150 to 170 ml.  $\text{CD34}^+$  progenitor cells were purified by magnetic cell sorting using the Super MACS System,  $\text{XS}^+$  enrichment columns and the  $\text{CD34}$  multisort kit (Miltenyi Biotec, Bergisch Gladbach, Germany). A second purification round was performed using  $\text{MS}^+$  enrichment columns (Miltenyi Biotec). Purified  $\text{CD34}^+$  cells were cultured in RPMI 1640 with glutamax I (Gibco BRL, Eggenstein, Germany), supplemented with 5% human AB serum (PAA Laboratories, Cölbe, Germany), GM-CSF (100 U/ml; Sigma, Deisenhofen, Germany) and TNF- $\alpha$  (5 ng/ml; Sigma) for 10–12 d at a density of  $2 \times 10^5$  cells/ml. Every other day, half of the culture solution was replaced with fresh cytokine-containing medium. Eighteen hours before vaccination, Id-derived peptides and  $\text{F(ab')}_2$  fragments were added to the DC preparations at a final concentration of 10  $\mu\text{g}/\text{ml}$ . Before subcutaneous (s.c.) injection, the cells were gently moved from the culture vessels using a cell scraper or by extensive rinsing with cold PBS. Subsequently, the cells were washed twice in 0.9% NaCl solution and resuspended in a final volume of 0.5 ml of NaCl solution.

**Vaccination procedure.** On day 0, the patients received Id-pulsed DCs by s.c. injection (Table 1). This first vaccination was followed by three booster injections with 100  $\mu\text{g}$  of Id peptides in combination with 100  $\mu\text{g}$  of GM-CSF

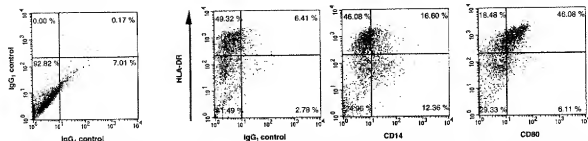


Fig 1. Flow cytometric analysis of DCs differentiated from CD34<sup>+</sup> progenitor cells in the presence of GM-CSF and TNF- $\alpha$ . DCs were stained with monoclonal fluorochrome-conjugated antibodies against CD14, CD80 and HLA-DR on day 10 of the differentiation.

(Leucomax; Sandoz, Frickenhausen, Germany) at days 14, 28 and 42 at the same injection site (s.c.). Patients 11 and 12 received booster injections with Id-pulsed DCs instead of Id peptides and GM-CSF. The patients did not receive additional therapies during the vaccination period.

**Cell surface marker analysis.** Cell surface markers were analysed by flow cytometry. Cells ( $2 \times 10^5$ ) were stained for 15 min at 4°C in PBS, 0.1% BSA, 0.01% NaN<sub>3</sub> with 20  $\mu$ l of phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)- or PerCP-labelled monoclonal antibodies: CD1a, CD3, CD4, CD8, CD14, CD16, CD25, CD34, CD45RO, CD56, CD69, anti-HLA-DR (Becton Dickinson, Heidelberg, Germany), CD40 (Immunex, Seattle, WA, USA) and CD80 (Ancell, Bayport, MN, USA). The samples were washed twice in 1 ml of PBS, fixed in PBS 0.5% formaldehyde and subsequently analysed on a FACSCalibur flow cytometer (Becton Dickinson).

**Purification of CD8/CD4 T-cells.** DC preparations were used in a syngeneic mixed lymphocyte reaction (MLR) to induce T-cell stimulation. T cells were purified by depletion of CD14<sup>+</sup>, CD19<sup>+</sup> and CD8<sup>+</sup> or CD4<sup>+</sup> cells from the leukapheresis material with paramagnetic microbeads (Miltenyi Biotec). Magnetic cell separation was performed according to the manufacturer's protocol and resulted in a CD4/CD8 T-cell-enriched population of >80% purity as examined by flow cytometry.

**Autologous MLR.** CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were stimulated with  $1 \times 10^4$  DCs in the absence or presence of antigen [tetanus toxoid: 40 LE/ml (Behring, Marburg, Germany); Id peptides and F(ab')<sub>2</sub> fragments, 10  $\mu$ g/ml]. T cells or DCs alone served as negative controls. The assays were carried out for 96 h in 96-well round-bottomed plates (Greiner, Frickenhausen, Germany). The cells were pulsed with 37 kBq of [<sup>3</sup>H]-thymidine (Amersham, Braunschweig, Germany) during the last 18 h of incubation and harvested using a Beckman cell harvester. The proliferation rate was measured by [<sup>3</sup>H]-thymidine incorporation on a liquid scintillation counter (Beckman, Düsseldorf, Germany).

**Detection of idiotype-specific antibodies by ELISA.** 96-well plates were coated overnight in PBS, pH 7.4, with Id F(ab')<sub>2</sub> fragments (10  $\mu$ g/ml, 4°C) washed with PBS, and non-specific binding sites were blocked with PBS, 1% BSA for 1 h at room temperature. The patients' sera were diluted in a 1:5 dilution cascade and incubated at 4°C overnight. Idiotype-specific antibodies were detected with horseradish

peroxidase (HRP)-labelled anti-human IgM or IgG antibodies (1:4000; SBA, Birmingham, AL, USA) and developed with ABTS solution, which was diluted to a concentration of 0.3 mg/ml in citrate buffer containing 0.3% H<sub>2</sub>O<sub>2</sub>. The OD was measured at 405 nm in an ELISA reader (Biermann DPC, Bad Nauheim, Germany).

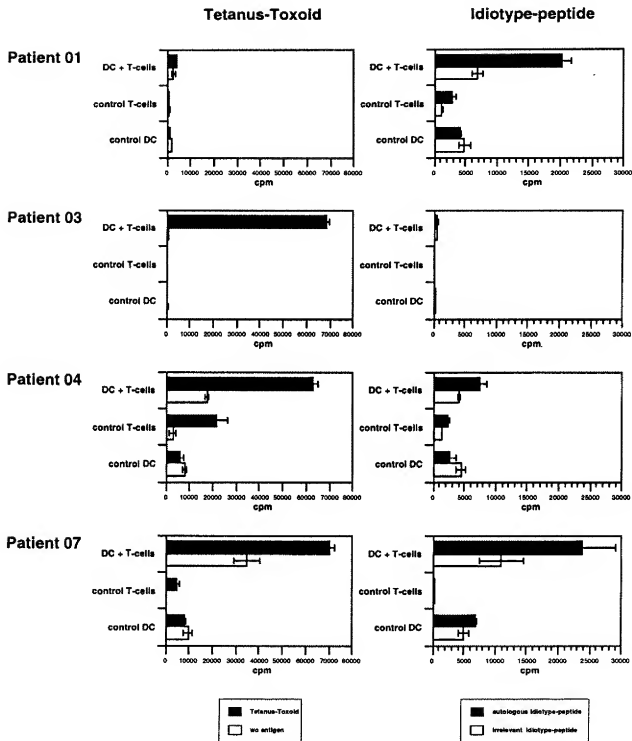
**Evaluation of idiotype-specific T cells by interferon (IFN)- $\gamma$  ELISpot assay.** Nitrocellulose HA S45 Millipore plates (Millipore, Eschborn, Germany) were coated (4°C) overnight with anti-IFN- $\gamma$  antibodies (clone 1-D1K, 10  $\mu$ g/ml; Mabtech, Nacka, Sweden) in carbonate/bicarbonate buffer. After washing with PBS, the remaining free binding sites were blocked using RPMI 1640 with glutamax 1 (Gibco BRL, Eggenstein, Germany) and 10% fetal calf serum (FCS; Gibco BRL). Subsequently,  $2 \times 10^5$  peripheral blood mononuclear cells (PBMCs) or purified T cells before and 8 weeks after vaccination (enriched by depletion) were stimulated in triplicate with  $2 \times 10^3$  DCs in the presence of autologous Id peptides and F(ab')<sub>2</sub> fragments (10  $\mu$ g/ml) or isotype-matched Id fragments from other patients with MM. T cells or APCs alone served as negative controls. After an incubation period of 48 h, the plates were washed with PBS and incubated for 2 h with anti-IFN- $\gamma$  biotinylated antibodies (clone 7-B6-1; Mabtech, Nacka, Sweden) at 37°C. Avidin alkaline phosphatase (dilution 1:2000) (Sigma) was added for 60 min after washing with PBS. After washing the plates in NaCl 0.9% solution (Delta-Pharma), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate solution (Sigma) was added, followed by incubation for 5–10 min. The analysis of the plates was done using a KS ELISpot analysis system (Carl Zeiss Vision, Echting, Germany).

**Detection of Ig in the serum of MM before and after immunization.** In order to determine the concentration of Ig, the patients' sera and urine were analysed by standard methods using the antisera for detection of Bence-Jones protein and the N antisera to human Ig (Behring, Marburg, Germany). Measurements were carried out on the Behring nephelometer BN100 according to the manufacturer's recommendations.

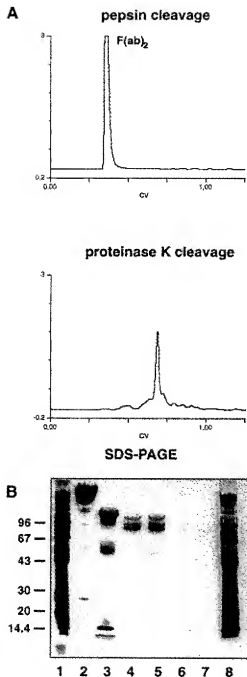
## RESULTS

### *In vitro generation of dendritic cells*

The enrichment of CD34<sup>+</sup> peripheral blood progenitor cells



**Fig 2.** CD4<sup>+</sup> T-cell stimulation assay with DCs differentiated from GM-CSF/TNF- $\alpha$ -stimulated CD34<sup>+</sup> progenitor cells in the presence of tetanus toxoid and idiotype peptides. Approximately 10<sup>5</sup> CD4<sup>+</sup> T cells from the study patients were stimulated with autologous DC-34 and tetanus toxoid or Id peptides before the vaccination therapy (T-cell/DC ratio 10:1; tetanus toxoid concentration 40 LE/ml; idiotype peptide concentration 10  $\mu$ g/ml; without (wo) antigen, cells in medium only). Representative stimulations are depicted from patients 01, 03, 04 and 07. The T-cell proliferation was analysed by [<sup>3</sup>H]-thymidine incorporation.



**Fig 3.** Preparation of idiotype  $F(ab')_2$  fragments and idiotype peptides. IgG paraprotein was isolated from the patients' serum using a protein G column.  $F(ab')_2$  fragments were obtained after pepsin cleavage and isolated via HPLC size exclusion.  $F(ab')_2$  fragments were digested further into peptides using proteinase K. The different isolation steps were evaluated by SDS-PAGE. (A) HPLC size exclusion shows the Id  $F(ab')_2$  fragments. (B) Id peptide fractions after proteinase K digestion. (C) SDS-PAGE: lane 1, size marker; lane 2, whole Ig; lane 3, Ig fragments,  $F(ab')_2$  and Fc after pepsin cleavage, lanes 4 and 5,  $F(ab')_2$  after HPLC size exclusion; lanes 6 and 7, peptide fraction after proteinase K digestion.

from the patients' leukapheresis material by magnetic cell sorting resulted in purification grades of 90–97%. The contamination by T cells, B cells, natural killer (NK) cells and monocytes did not exceed 10%.

After the *in vitro* differentiation period of 10–12 d in the presence of GM-CSF and TNF- $\alpha$ , the resulting DCs were analysed for the expression of characteristic DC surface markers. The *in vitro*-differentiated DCs displayed a typical DC phenotype with the expression of CD1a, CD40, CD80, HLA-DR and a dim expression of CD14 on 10–18% of the cells. Figure 1 depicts the expression of CD80, HLA-DR and CD14.

#### Functional analysis of CD34-derived DCs (DC-34)

Before the Id vaccination procedure, CD4<sup>+</sup> T-cell stimulation assays were carried out in the presence of Id peptides and tetanus toxoid to evaluate the *in vitro* stimulatory capacity of DCs and a pre-existing Id-specific autologous T-cell response. Figure 2 depicts the antigen-specific T-cell proliferation in patients 01, 03, 04 and 07. The *in vitro*-differentiated DC-34 stimulated tetanus-specific T cells in all patients except patient 01, who has never been immunized against tetanus toxoid. A pre-existing anti-idiotypic T-cell response was measured in patients 01 and 07 after stimulation of peripheral blood T cells with autologous id-pulsed DC-34 (Fig 2). A slight idiotype-specific T-cell response was also observed in patient 04. The observed proliferation was found to be specific for the autologous myeloma Id, as irrelevant Id fragments from isotype-matched MM patients did not induce a significant T-cell proliferation. The proliferation assays for patient 01 were done with control Id peptides from patient 04 and vice versa, as both patients had a IgG  $\kappa$  myeloma. Assays for patient 07 were set up with control Id peptides from patient 08 (both IgG  $\lambda$  myeloma).

#### Isolation of idiotype $F(ab')_2$ fragments and Id peptides

Idiotype-derived  $F(ab')_2$  fragments were purified from a pepsin digestion of isolated paraprotein from the patients' serum, whereas light chains (patients 03 and 10) were isolated from urine. Subsequent analysis by SDS-PAGE revealed complete Ig degradation into Fc and  $F(ab')_2$  fragments. These were then separated by size-exclusion chromatography for further digestion of the  $F(ab')_2$  into Id peptides by proteinase K digestion (Fig 3A and B). Purity and size were confirmed further by electrophoresis and reverse-phase chromatography (Fig 3C).

#### Clinical effects and toxicity

Ten patients with advanced MM were vaccinated with autologous *in vitro*-differentiated DCs that had been pulsed with tumour-idiotype fragments. In addition, three boost immunizations were carried out every 2 weeks with a combination of Id peptides and GM-CSF. Six out of 10 treated patients showed osteolytic lesions before vaccination therapy and were in clinical stage III. Patient 02 was in clinical stage II with an increased concentration of IgG in the serum varying from 50 to 70 g/L. Increased concentrations of paraprotein were measured in the sera of patients

01, 04, 05 and 11 before Id vaccination. All patients had received various cycles of chemotherapy up to 2 months before vaccination therapy (see Table I).

Table II summarizes the observed therapy-related immunological and clinical effects. Three months after vaccination, patient 10 had a reduction in plasma cells in the bone marrow from 8–10% to 4% and was staged as stable disease. Nine patients had progressive disease evaluated 8 weeks after the application of the DCs. Patients 03, 05, 07, 08 and 11 had new osteolytic lesions 2 months after vaccination therapy. Progressive disease was detected in the bone marrow of patient 01. Patient 02 was still in clinical stage II 8 weeks after therapy, with a paraprotein serum concentration of < 70 g/l. No therapeutic side-effects could be observed in the vaccinated patients. According to the National Cancer Institut (NCI) classification, they were therefore staged as level I.

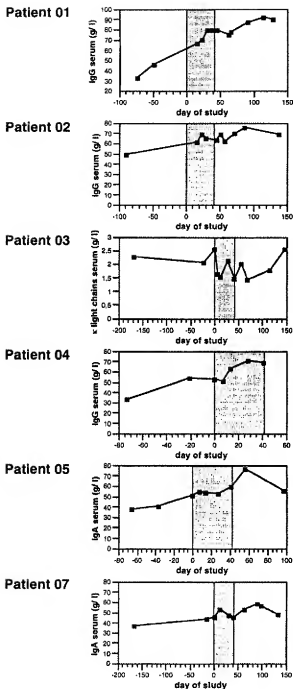
#### *Ig level in the serum of MM patients before and after immunization*

The monoclonal paraprotein is an important parameter for evaluating the activity of malignant plasma cells in MM. Here, the paraprotein concentration in the patients' sera was measured by nephelometric analysis. Figure 4 shows the concentration of complete immunoglobulin or light chains in the sera of patients 01, 02, 03, 04, 05 and 07 in the course of the therapy.

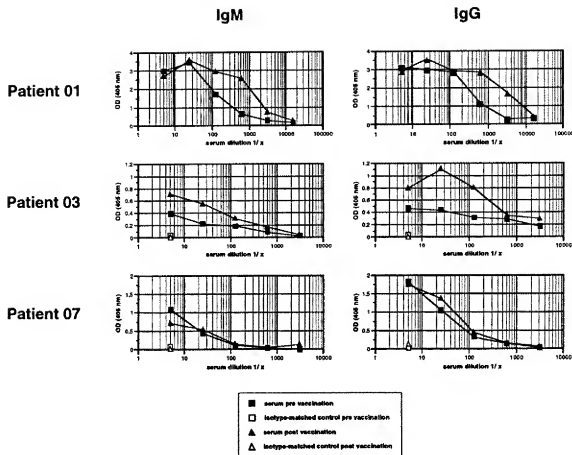
The analysis for patient 01 revealed an increasing paraprotein concentration 70 d prevaccination until 30 d after DC vaccination. For the following 40 d, the Ig concentration remained stable at  $\approx 78$  g/l. From day 70, the Ig concentration showed an increasing course again. A similar observation was made for patient 05, with a stable serum level from day 5 to day 25 during the vaccination period. The concentration of  $\lambda$  light chains in the serum of patient 03 showed no significant changes during the monitoring period. Increasing paraprotein concentrations during or after the vaccination period were analysed in patients 02 and 07. No therapy-related effects were observed in the sera of the six remaining patients.

#### *Anti-idiotypic antibodies in the patients' sera before and after vaccination*

To evaluate the activation of an idiotype-specific humoral response, the patients' sera were analysed by idiotype-specific ELISA. The samples were obtained before and 6 weeks after vaccination and were diluted in a serial dilution of 1:5. An increase in anti-Id antibody titres was observed in patients 01, 03 and 10. The titres calculated at half-maximal OD revealed a 10-fold increase in IgM and an eightfold increase in IgG idiotype-specific antibodies for patient 01 (Fig 5), and a 10-fold increase in IgM and a 15-fold increase in IgG idiotype-specific antibodies in patient 03. An increase in IgM idiotype-specific antibodies was observed in patient 10 (data not shown). The control experiments with coated isotype-matched irrelevant Id F(ab')<sub>2</sub> fragments showed no cross-reactivity to allogeneic Id protein pointing towards pre-existing levels of idiotype-specific antibodies. There was no increase in the



**Fig 4.** Light-chain and Ig serum level in the study patients. Light-chain or Ig serum concentrations (g/l) were analysed in the vaccinated patients by nephelometric standard procedures in the course of the idiotype vaccination. The vaccination time point with the idiotype-pulsed DCs is termed 'day 0'. The vaccination period is marked in grey. Graphs are shown from patients 01, 02, 03 ( $\lambda$  light-chain myeloma), 04, 05 and 07.



**Fig 5** Anti-idiotypic antibodies in the serum of the study patients before and after vaccination detected by ELISA. The serum from the vaccinated patients was examined in serial dilution for the presence of anti-idiotypic antibodies of the IgM and IgG subtype before and 6 weeks after immunotherapy. The corresponding controls with isotype-matched allogeneic Id protein are marked by unfilled symbols. Representative results are shown from patients 01, 03 and 07.

idiotype-specific antibody titre in patient 07 (Fig 5) and the remaining evaluable patients.

#### *Idiotypic-specific T-cells in the peripheral blood before and after vaccination*

The frequency of idiotype-specific T cells in the peripheral blood was analysed by IFN $\gamma$ -specific ELISpot analysis. The samples were obtained before and 8 weeks after vaccination. To analyse the vaccine-related idiotype-specific immune response, the T cells or PBMCs were stimulated with autologous Id-derived peptides or Id control peptides from isotype-matched myeloma patients. Figure 6A shows the mean number of spots resulting from PBMCs of patients 03 and 07 that were stimulated by Id peptide and irrelevant peptide. In patient 03, the mean number of specific spots increased from 14 spots before vaccination to 26 spots after vaccination, whereas the irrelevant peptides did not show a comparable increase in T-cell reactivity. Similar results were obtained in patient 01 (data not shown). In order to analyse the MHC restriction of the observed T-cell response, purified CD4- and CD8-positive cells from patients 11 and 12 were

stimulated with autologous DCs and peptides (Fig 6B). After vaccination, patient 11 showed an increase in idiotype-specific CD8 T-cell spots from 21.5 to 40. These results indicated a major increase in the class I restricted T-cell response for patient 11. The analysis for patient 12 revealed an activation of IFN $\gamma$  secreting idiotype-specific CD4 T cells with a mean of 11 spots before and 45.5 spots after vaccination.

Overall, four patients showed increased idiotype-specific T-cell activity after vaccination. No effects regarding the frequency of idiotype-specific T cells were measured in the other patients analysed.

#### DISCUSSION

Conventional therapy, except for high-dose chemotherapy and stem cell transplantation, has no curative perspective for myeloma patients to date. Alternative immunotherapeutic approaches can be targeted towards the TAAs represented as the monoclonal myeloma Ig. The intact Ig is a weakly immunogenic antigen, which requires optimal

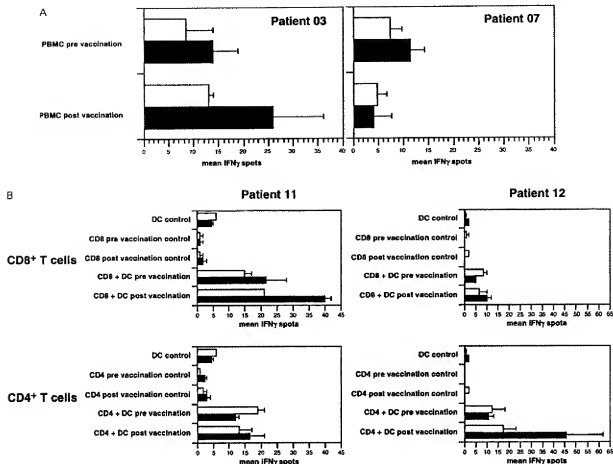


Fig 6. Idiotypic-specific T-cell activity before and after vaccination. (A) Idiotypic-specific IFN $\gamma$  ELISpot assays were carried out in triplicate before and after vaccination with PBMCs in the presence of autologous Id (black bars) or control Id from an isotype-matched MM patient (white bars). The means of IFN $\gamma$ -specific spots are shown with the standard deviation. Representative results from patients 03 and 07. (B) In patients 11 and 12, idiotypic-specific IFN $\gamma$  ELISpot assays were carried out in triplicate before and after vaccination with purified CD4 or CD8 T cells and DCs in the presence of autologous Id (black bars) or control Id from an isotype-matched MM patient (white bars).

presentation by professional APCs to elicit an immune response. Application of TAAs alone might lead to tolerance and further tumour progression (Toes *et al.* 1996). The use of professional APCs in immunotherapy can be a suitable tool to overcome the tumour-specific tolerance and tumour growth (Toes *et al.* 1998). As shown by Flamand *et al.* (1994) in a lymphoma mouse model, protective immunity was only induced after the application of Id-pulsed DCs. For the first time in humans, Hsu *et al.* (1996) described the use of Id-pulsed DCs in a vaccination approach of four patients with low-grade non-Hodgkin lymphoma. After vaccination, they observed an increase in tumour-reactive cytotoxic T cells. In addition, one patient experienced a complete tumour regression, and a second patient had partial tumour regression. These observations underlined the importance of DCs as natural adjuvants in lymphoma immunotherapy of humans.

Hence, in the present clinical trial, Id vaccination was mediated by a defined amount of CD34-derived DCs pulsed

with myeloma Id fragments to induce a *de novo* or augment an ongoing anti-idiotypic immune response, as the amount of GM-CSF recruitable APCs *in situ* is not predictable. The study monitoring focused on the evaluation of idiotypic-specific cellular and humoral immune responses. After vaccination, an increase in anti-idiotypic IgM or IgG serum titres was observed in three out of 10 analysed MM patients, despite high paraprotein serum concentrations. It seems likely that anti-idiotypic antibodies in the patients' sera exist in the form of immune complexes bound to the serum paraprotein. Nonetheless, a detection of anti-Id antibodies in an ELISA experiment was possible. To reach a biochemical equilibrium, the paraprotein-bound anti-Id antibodies could bind to the coated Id protein whenever the serum was diluted. To enhance this process, a prolonged serum incubation time (overnight at 4°C) was found to be necessary.

The observed increase in anti-idiotypic IgM antibodies points towards a *de novo* anti-idiotypic immune response.

whereas Id-binding antibodies of the IgG isotype indicate the involvement of an idiotypic-specific T-helper response. Activation of a specific humoral immune response after the administration of DCs is in agreement with data obtained in mouse models, in which antigen-pulsed DCs induced idiotypic-specific antibody responses (Sornasse *et al.* 1992). The mechanism underlying the anti-idiotypic antibody induction could reflect a T-cell-mediated activation of B cells or a direct modulation of B-cell activity by DCs, a model discussed recently by Dubois *et al.* (1995, 1997). In MM, the importance of an idiotypic-specific humoral immune response was demonstrated by the application of anti-idiotypic antibodies, eliciting passive immunity against myeloma cells in an animal model (Chen *et al.* 1976).

Before the vaccination, we confirmed earlier findings from another group concerning the pre-existence of anti-idiotypic T cells in MM (Fig 2) (Osterborg *et al.* 1991, 1995; Yi *et al.* 1997b), the activation and expansion of which would be desirable during immunotherapy. Indeed, the analysis of idiotypic-specific T cells before and after vaccination by IFN $\gamma$  ELISpot assays revealed an increase in idiotypic-specific T-cell responses in four out of 10 analysed patients. The activation of IFN $\gamma$ -producing T-cell CD4 Th1 or CD8<sup>+</sup> CTL subsets is an important aim of tumour-specific vaccination strategies. Th1 T cells were shown to play a major role in protection against tumours in a mouse model (Gabrilovich *et al.* 1996; Zitvogel *et al.* 1996). Using an Id vaccine, Nelson *et al.* (1996) showed that the increase in idiotypic-specific CTLs after vaccination correlated with a better clinical prognosis in patients with NHL. To determine whether the increase in idiotypic-specific T-cell activity in our patients was mainly CD4 or CD8 mediated, ELISpot assays for patients 11 and 12 were carried out with highly purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells. An increase in the idiotypic CD8<sup>+</sup> T-cell reactivity for patient 11 was detected, whereas the analysis for patient 12 revealed an increase in idiotypic-specific IFN $\gamma$ -secreting CD4<sup>+</sup> T cells. This points towards the activation of idiotypic-specific CTLs and idiotypic-specific CD4<sup>+</sup> T cells by the DC vaccination. Indeed, DCs are capable of priming extensive CD8 CTL responses in CD4 T-cell-depleted lymphocytes (Young & Steinman, 1990; Bhardwaj *et al.* 1994). Furthermore, several groups have reported on the activation of Th1 T cells mediated by DCs (Heufler *et al.* 1996). Here, the observed CD8 or CD4 T-cell increase in patients 11 and 12 has to be DC mediated as well and cannot be induced by Id plus GM-CSF administration, as these patients received DCs instead of GM-CSF for the booster immunizations. In the other patients, the administration of Id fragments in combination with GM-CSF for boosting might have played a role, in that GM-CSF stimulates DCs and may increase their capacity to present tumour Ag (Grabbe *et al.* 1995).

To evaluate the effects of the DC-based vaccination on the patients' peripheral blood T-cell compartment, cell surface marker expression was monitored by flow cytometry (data not shown). At several time points during the vaccination, we monitored the CD4 and CD8 T-cell counts. In two patients, FACS analysis of peripheral blood T cells revealed an increase in T-cell activation markers on CD4 and/or CD8

cells during the DC vaccination period. In patient 11, the expression of CD25 and CD69 on CD8 T cells showed an increase, which lasted up to 220 d after vaccination. This observation suggests that a DC-based idiotypic vaccination can influence the activation status of peripheral blood T cells.

Besides the evaluation of idiotypic-specific immune responses, we monitored the clinical course of the disease. One patient experienced a reduced infiltration of plasma cells in the bone marrow, whereas the remaining patients had a progressive course of disease. Despite this poor clinical response, we observed a biological response in five out of 11 patients after vaccination. One reason might be the advanced disease stage of the vaccinated patients and the resulting high tumour load.

The aim of this phase I clinical trial was to evaluate the safety and feasibility of a DC-based myeloma-specific vaccination in patients pretreated with conventional therapy. It could not be anticipated that treatment of severely ill stage III myeloma patients with DCs would result in major clinical benefits, especially as the bone marrow, being the predominant site of tumour growth, loses its ability to give rise to a normal pool of immunological active cells with disease progression. Early-stage myeloma patients are more likely to experience a clinically relevant antitumour response after DC vaccination and are therefore a preferred patient group in follow-up studies.

Recently, several groups have focused on non-specific T-cell activation in MM, such as the treatment with IL-2 (Peest *et al.* 1996) or anti-CD3 antibodies (Borriore *et al.* 1996), with limited immunological and clinical outcome. A more specific immune response of short duration was achieved by using TAAs as vaccines. After vaccination with Ids using the autologous M-component alone, Bergenbrant *et al.* (1996) achieved short-term anti-idiotypic T- and B-cell responses. In a follow-up study, Osterborg *et al.* (1998) applied M-components in combination with GM-CSF to five patients with IgG myeloma. They observed idiotypic-specific T-cell responses that were predominantly MHC class I restricted and a transient increase in IgM anti-idiotypic antibodies. The strategy of using GM-CSF in the primary vaccination procedure focused on the attraction of APCs to the site of injection, although the amount of recruitable APCs *in situ* is difficult to evaluate. GM-CSF has several functions. On the one hand, it stimulates DCs and may increase their capacity to present tumour Ag (Grabbe *et al.* 1992, 1995). Furthermore, GM-CSF is involved in the maturation of DCs. Initial experiments by Disis *et al.* (1996) demonstrated that repeated injections of GM-CSF into the skin of rats resulted in an increase in MHC class II<sup>+</sup> cells. On the other hand, growth-stimulating effects of GM-CSF on human myeloma cells have been described *in vitro* (Zhang *et al.* 1990), an observation that might be strengthened by the findings of Celis *et al.* (1992) who reported on the extramedullary progression of MM after GM-CSF treatment. Therefore, a vaccination using DCs without the additional application of GM-CSF would be preferable for future studies.

Differences observed in clinical outcome between the present study and the report of Osterborg *et al.* (1998) could

result from a different group of patients. While in our study, nine out of 10 patients were clinical stage III and formerly underwent multiple chemotherapies, Osterborg *et al* (1998) vaccinated partially untreated MM patients with clinical stage IIa. Recently, Reichardt *et al* (1999) reported on the vaccination of myeloma patients with idiotype-pulsed DCs after autologous stem cell transplantation. This group detected the activation of idiotype-specific T cells in two out of 12 patients who maintained a pre-existing CR after stem cell transplantation, whereas the other patients had SD ( $n = 1$ ), PD ( $n = 6$ ) or died ( $n = 3$ ). These data indicate the importance of a myeloma-specific T-cell response regarding a favourable clinical outcome. The lack of a clinical response in our patients who reached a myeloma-specific T-cell response might be the result of their advanced stage of disease.

Taken together, our Id vaccination approach demonstrated that DCs could serve as a natural adjuvant for the induction of idiotype-specific immunity in patients with advanced MM. Despite the advanced stage of the treated patients, immunological responses and clinical effects were observed in some cases. Future studies will focus on the treatment of clinical stage I-IIa patients with DCs, but without the application of GM-CSF to eliminate the risk of side-effects.

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